IRON, INFECTION, AND MALNUTRITION: AN EXPLORATION OF CHILDHOOD ANEMIA IN A PERUVIAN PERI-URBAN COMMUNITY

Achsah Dorsey

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Approved by

Amanda Thompson,

Mark Sorensen,

Paul Leslie,

Elizabeth Miller,

Margaret Bentley

ABSTRACT

Achsah Dorsey: Iron, Infection, and Malnutrition: An Exploration of Childhood Anemia in a Peri-Urban Community in Lima, Peru
(Under the direction of Amanda Thompson)

Anemia remains common worldwide among young children, despite numerous public health interventions and the availability of inexpensive iron supplements. Peru manifests some of the highest rates of anemia in South America, roughly comparable to countries in sub-Saharan Africa, where anemia rates tend to be highest. Several initiatives by the Peruvian Ministry of Health have attempted to reduce levels of anemia, however, national rates of anemia have remained high and even increased in some areas.

One potential explanation for persistently high rates of anemia is that they represent an evolutionary adaptation for combatting infectious disease since low levels of iron availability impede the survival and reproduction of pathogens. The Optimal Iron Hypothesis proposes that iron deficiency protects against debilitating levels of infectious disease and that an individual's optimal iron status is contingent on their particular environmental context. The body must therefore strike a balance between iron withholding and iron availability in order to protect against infection while avoiding compromised immune function. This project expands on existing scholarship by exploring anemia in 102 pre-school aged children living in a community within San Juan de Lurigancho.

Analyses from this study establish predictors of childhood anemia and response to iron supplementation, explore the relationship between energetics and immune response, as well as identify the role of intestinal microbiota diversity on recovery from anemia. The associations

identified between child growth patterns coupled with maternal perceptions of child body size, household composition, and seasonality with iron status indicate the importance of including caregiver, household, and environmental factors in addition to individual-level variables in studies of childhood anemia. While obesity has been shown to increase inflammation and decrease iron absorption, results from my study complicate this narrative. Analyses show different patterns of response to iron supplementation between children with high and low central adiposity and total body fat, this demonstrates how fat distribution can impact immune function and nutritional status. This work also identifies the intestinal microbiome as an underlying pathway linking nutritional deficiencies and disease ecology through observed differences in gut microbiota and taxa in pre- and post- iron supplementation samples, demonstrating the need to include gut health indicators in medical and nutritional interventions.

“Adapt and Overcome”
The unofficial motto of the United States Marine Corps

To those who have had to adjust to new conditions to better live in their specific context
whether fast or slow, consciously or unconsciously

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BMI	Body mass index
C	Celsius
CI	Confidence interval
CRP	C-reactive protein
DBS	Dried blood spot
dL	Deciliter
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
ENDES	*Encuesta Demográfica y de Salud* (Demographic and Health Survey)
FS	Fingerstick
g	Grams
HAZ	Height-for-age z-score
Hb	Hemoglobin
HFIAS	Household Food Insecurity Access Scale
Hz	Hertz
IIN	*Instituto de Investigación Nutricional* (Institute of Nutrition Research)
Il-6	Interleukin 6
INEI	*Instituto Nacional de Estadistica e Informatica* (National Institute of Statistics and Information)
IRB	Institutional Review Board
L	Liter
mg	Milligrams
mL	Mililiter

mm	Millimeter
mM	Millimolar
MNP	Micronutrient powders
n	Total number of observations
NaOH	Sodium hydroxide
ng	Nanogram
NGO	Non-governmental organization
OIH	Optimal Iron Hypothesis
OR	Odds ratio
OTUs	Operational taxonomic units
PANS	Pediatric acute-onset neuropsychiatric syndrome
PCR	Polymerase chain reaction
PD	Phylogenetic diversity
rDNA	Ribosomal deoxyribonucleic acid
rpm	Rotations per minute
SD	Standard deviation
sTfR	Serum transferrin receptor
TNF-α	Tumor necrosis factor alpha
TSF	Triceps skinfold
uL	Microliter
um	Micrometer
UNC	University of North Carolina
V	Venipuncture

WAZ	Weight-for-age z-score
WIC	Women, Infants, and Children
WHO	World Health Organization
WHtR	Waist-to-height ratio

CHAPTER 1. INTRODUCTION

Prior to the nineteenth century, when people observed the symptoms associated with anemia they believed they were caused by unrequited passion (Farley and Foland 1990). Artists and playwrights explored these romantic connections in their work by invoking the pallor associated with anemia. Shakespeare, for example, described characters that had been disappointed by love as "smitten with green sickness" (Farley and Foland 1990). With the advent of modern medicine in the 20th century, health researchers discovered that anemia is caused by low levels of hemoglobin, a protein vital to the transportation and storage of oxygen in organs and tissue. Since this discovery, a variety of factors have been identified that influence a person's hemoglobin level. Iron deficiency is considered the leading cause of anemia globally (Stoltzfus et al. 2004) and established risk factors for this disease include physiological (e.g. age and sex), nutritional (e.g. low iron consumption), and pathological (e.g. blood loss, inflammation, and malabsorption) conditions (Lopez et al. 2016).

Despite the identification of proximate and distal causes, however, anemia remains a widespread public health problem. Anemia affects an astounding 1.62 billion people, almost 25% of the world's population, with reproductive-age women and pre-school-aged children carrying a disproportionate amount of the burden (World Health Organization [WHO] 2009). Severe anemia is a both a direct and indirect contributing factor to morbidity and mortality (Macgregor 1963; Scholl & Hediger 1994; Bothwell & Charlton 1981). Anemia is a risk factor for maternal mortality, in Latin America 7.26% of maternal deaths can be attributed to severe iron deficiency (Brabin et al. 2001). Pregnant women with anemia are also at an increased risk of having a preterm birth, which, in turn, is linked to several serious health outcomes in infants, such as respiratory disease syndrome, stunting, brain damage, and anemia (Alcázar 2013). Childhood anemia can cause delayed and decreased cognitive and physical development and function (Beard 2008; Stoltzfus et al. 2004a). The consequences of these symptoms are associated with loss of productivity, including reduced work capacity, cognitive impairment, and increased susceptibility to infection (Balarajan et al. 2011).

Due to concerns about anemia's negative impact on child development, global health institutions recommend iron supplementation and fortification for all children in populations with a high prevalence of anemia (Sazawal et al. 2006; WHO 2002; Stolzfus and Dreyfuss 1998). Supplementation and fortification have been proven to be effective public health interventions to reduce anemia rates (Thompson et al. 2013; Zimmermann and Hurrell 2007; Baltussen et al. 2004). A comprehensive review of the efficacy of iron supplementation concluded that the majority of randomized-control-trials investigating the effectiveness of iron supplementation in children report significant increases in hemoglobin concentration and other iron status indicators as well as reduced anemia prevalence (Iannotti et al. 2006). Where iron supplementation has not been effective in reducing anemia individually, experts suggest investigating poor compliance (Galloway and McGuire 1994) and malabsorption (Lopez et al. 2016).

However, despite the reported benefits of iron supplementation, public health officials have raised concerns about the risks associated with iron supplementation among preschoolers. Recent systematic reviews of randomized, controlled trials of iron supplementation conclude that supplementation moderately increases the risk of diarrheal disease (Gera and Sachdev 2002) and malaria (Oppenheimer 2001). Since 2002, several more studies documented an association between iron fortification and increased diarrhea prevalence (Zlotkin et al. 2013, Soofi et al. 2013, Chang et al. 2010, Richard et al. 2006). Additional studies report an association between iron level and infection caused by malaria parasites with (Sazawal et al. 2006) and without (Nyakeriga et al. 2004) iron supplementation. These results demonstrate the importance of considering rates of infection and disease patterns when designing anemia intervention programs.

EVOLUTIONARY MEDICINE

One explanation for the negative effects of iron supplementation on health comes from the field of evolutionary medicine, which proposes that some manifestations of disease may act as adaptive defenses against other types of disease (Ewald 1994, Williams and Nesse 1991). Infection with common pathogens has been linked to higher C-reactive protein (CRP), an inflammatory acute-phase protein commonly used as a marker of systemic inflammation, levels in children (Dowd et al. 2010) and adults (Nazmi et al. 2010; Zhu et al. 2000), with greater risk of inflammation seen with increasing pathogen burden. Inflammation is one of the first responses of the immune system to infection and signals biological systems to sequester iron, reduce iron absorption, and decrease erythropoiesis (the production of red blood cells) which causes a decrease in serum hemoglobin, resulting in anemia (Weinberg 1992). Decreasing the amount of circulating iron and iron absorption restricts the availability of iron to pathogens, inhibiting pathogen growth, proliferation, and virulence (Nemeth and Ganz 2006).

The Optimal Iron Hypothesis

The Optimal Iron Hypothesis (OIH) states that an individual's optimal iron status is contingent on local disease patterns. In environments with high levels of endemic infectious disease, restricted iron intake may protect against infection (Wander et al. 2009). This hypothesis builds on previous evolutionary medicine theory to explain the relationship between iron and infection, arguing that anemia may not always be pathological and may instead act as an adaptive defense against infection. In this case, while childhood anemia impairs growth and cognitive development, in areas with high disease ecology prevalence anemia may be beneficial as it reduces bacterial proliferation and virulence (Figure 1.1).

In the same paper that proposed the OIH, Wander et al. (2009) examined the association between infection (CRP) and iron status (serum transferrin receptor [sTfR] and zinc protoporphyrin to heme ratio) among 270 school-age children in Kenya. When controlling for age and triceps skinfold thickness, the authors found evidence that an iron replete condition increases an individual's odds of infection and that clinical iron deficiency may therefore protect against infection. The authors did not find evidence that the odds of infection change with the degree of iron deficiency. This study showed support for their proposed hypothesis, but the small sample size and cross-sectional design are not ideal for showing causation.

Hadley and DeCaro (2015) tested the predictions of the OIH using a nationally representative sample of 1164 Tanzanian children aged 6-59 months living in both rural and urban settings using levels of CRP as a marker for infection and measurements of sTfR and hemoglobin to assess iron levels. The authors report that non-anemic low iron levels (normal hemoglobin, but below normal serum transferrin receptor) were not associated with a lower likelihood of infection compared to iron replete children. These findings do not support the OIH as it was initially formulated, Hadley and DeCaro propose that it may be more fruitful to investigate iron regulation as an allostatic system that responds to infection adaptively as opposed to expecting an optimal pre-infection value.

Dorsey et al. (2018) report additional support for this claim through secondary data analysis of the association between hemoglobin levels and morbidity among children living in Canto Grande, a peri-urban community located on the outskirts of Lima, Peru.

The authors used risk ratios to test whether lower hemoglobin status, assessed using the HemoCue B-Hemoglobin System, was associated with an increased relative risk of morbidity symptoms compared to non-anemic status, controlling for infant age, sex, weight for height z-score, maternal education, and repeated measures in 515 infants aged 6-12 months. Infants with fewer current respiratory and diarrheal morbidity symptoms had a lower risk of low hemoglobin compared to participants who were not anemic (p-value<.10). Infants with fewer current respiratory infection symptoms had a statistically significant (p-value<.05) reduction in risk of moderate anemia compared to infants who were not anemic. In this study, morbidity status was not predictive of hemoglobin status over a six-month interval period, but anemia status was shown to be associated with current morbidity symptoms.

LIFE HISTORY THEORY

Human physiological systems, like the immune system, are products of natural selection, designed to develop and function in individuals that are in turn integral components of their physical and social environments (Williams and Nesse 1991). Growth, reproduction, and maintenance are key evolutionary traits determining survival and fitness. Maintenance is conducted through immunology, a costly defense system in terms of the resources it needs in order to perform its functions and the consequences it has for wellbeing when immune processes are misdirected (McDade 2003). A fundamental model to understanding these developmental, reproductive, and immunological strategies is life history theory (LHT), a comparative evolutionary framework that seeks to explain biological aspects of an organism in reference to their life histories (Charnov 1993; Stearns 1992). Human ecological immunology is a population-level adaptationist perspective that uses LHT and ecology in order to understand immune function (McDade 2003). This approach is useful for understanding optimal iron level since life stages have different energetic trade-offs.

Essential to LHT is the notion of trade-offs, it is assumed that energy is limited and that energy must be allocated to the essential evolutionary traits. Energy spent on one function is no longer available for the other (Hill and Hurtado 1996). These types of trade-offs occur at multiple levels, genetically, developmentally, or more immediately (Lasker 1969), allowing for the ability to respond to ecological pressures within a range of plasticity (Hill and Hurtado 1996). The immune system is essential for maintenance and has significant inevitable costs (Sheldon and Verhulst 1996). Trade-offs occur with anemia, withholding iron can protect against infection but can also cause decreased cognitive function and physical performance as well as death. Life stage and ecological factors play major roles in determining the costs of these trade-offs.

Each life stage is characterized by different energetic demands. Adulthood, defined as the attainment of reproductive maturity, requires a balance of energetic demands for reproduction and maintenance. The energetic costs of reproduction differ dramatically between the sexes. Though men have a higher somatic maintenance cost than women due to body mass and the higher proportion of skeletal muscle (Bribiescas 2001), gestation and lactation require significant energy and time. Reproductive potential is sensitive to environmental variation in both energy availability and iron. Evidence suggests that iron is an important resource for egg development and when iron stores are low women may have reduced fecundity (Chavarro et al. 2006).

Pregnant women are at risk for anemia because of changes in hemodilution and transfer of iron from mother to fetus. Maternal anemia is related to many negative prenatal and postnatal outcomes (Steer et al. 1995, Bondevik et al. 2001, Levy et al. 2005). Several researchers believe that anemia during pregnancy represents an evolutionary mechanism that benefits maternal and fetal health (Weinberg 2010) while others suggest that populations may have developed dietary strategies, such as meat avoidance, to reduce iron intake and absorption (Fessler 2002). The reproductive iron withholding hypothesis posits that maternal-offspring prenatal and postnatal iron transfer systems have evolved to minimize risk of infection in offspring but also raises mothers' risk of poor reproductive outcomes (Miller 2016).

Infants (children between 0-2 years) experience intense energetic demands due to growth, unregulated immune activity, and even an active reproductive axis (McDade 2003). These competing demands result in severe trade-offs. Specific immune defenses are immature and the risk of mortality due to infectious disease is high. In some cases, breastfeeding offers a partial solution to an infant's energetic demands by limiting the severity of pathogen exposure, strengthening immune defenses, and restricting iron consumption (Quinn 2014). Low iron in breast milk may also have evolved to protect infants from oxidative stress in their intestines due to excess iron (Collard 2009).

Infancy also provides the immune system with antigenic input, which tailors the immune system to the potential pathogen exposure unique to the infant's environment. The hygiene hypothesis is one example of how the environment can shape immune system development. This theory proposes that the absence of infectious disease early in life may predispose children toward the development of atopic disease (Strachan 1989).

While infectious disease mortality risk in children between the ages of two and five is lower than in infancy, it is still significantly higher than older children and adolescents, indicating the vulnerability of pre-school-aged children. Childhood is characterized by slow and steady growth with delayed reproduction maturation (Bogin 1999). Like in infancy, the continued building of specific immune defenses to pathogens is important for survival. Children's mobility and increased independence exposes them to more antigens, resulting in the steady increase of memory cell lymphocytes (McDade 2003). Due to the naivety of the immune system in infancy and childhood, the nonspecific immune response (such as iron withholding) is incredibly important in the defense against infection.

Life history theory offers a framework for investigating the benefits and risks of childhood anemia, as energy devoted to immune defense can't be allocated to growth and development (Stearns 1992; Charnov 1993). The tension between allocating limited resources to immune defense or growth differs for those living in energy-rich environments versus those living in energy-poor contexts. Children with reliable access to nutrients are able to replenish the costs of immune activation while children experiencing undernutrition have more limited energy to devote to growth and consequently exhibit impaired immunity (McDade et al. 2008). The simple comparison between energy-rich and energy-poor environments is challenged by a global rise in overnutrition and urbanization, resulting in the dual burden of disease. While fat represents stored energy that is utilized for metabolic processes associated with growth and immune function (Kuzawa 1998), higher body weight and BMI have been associated with chronic inflammation in adults and children (Thompson et al. 2015; Choi et al. 2013; Dowd et al. 2010; Cook et al. 2000). Additional studies report that the association between central adiposity and pro-inflammatory markers may be particularly strong in younger populations (Fransson et al. 2010; Nguyen et al. 2009). This suggests that high energy stores (in the form of body fat) may not always be beneficial.

DUAL BURDEN OF DISEASE

Research suggests that both chronic pathogen exposure and high levels of adiposity activate pro-inflammatory pathways (Thompson et al. 2015; Vahdat et al. 2012; McDade et al. 2008a), thus overweight and obesity may also influence the efficacy of iron supplementation programs. In countries undergoing rapid dietary and lifestyle changes, obesity exists alongside illnesses associated with undernutrition, a phenomenon known as the 'dual burden of disease'

(Popkin, Adair, Ng 2012). A common manifestation of the dual burden in individuals is the co-occurrence of overweight and anemia. The co-occurrence between the two is attributed to increased iron requirements among overweight individuals (Yanoff et al. 2007) or physiological changes associated with overweight that influence iron absorption and utilization, such an increase of inflammatory acute-phase proteins, like CRP (Cheng et al. 2012). High levels of these proteins cause inflammation, triggering an innate immune response and increases one's risk for anemia due to iron sequestration and reduced iron absorption.

While the inverse correlation between adiposity and iron-level was established in the early 1960s (Seltzer et al. 1963; Wenzel et al. 1962), more recent evidence of the association between overweight and anemia has been mixed. Some studies report that higher body mass index (BMI) results in increased risk for iron deficiency and anemia among children and adolescents in both high income and transitioning settings (Aberli et al. 2011; Eftekhari et al. 2009; Zimmermann et al. 2008; Nead et al. 2004). Other studies have observed lower rates of anemia in women and children experiencing overnutrition (Kroker-Lobos et al. 2011; Eckhardt et al. 2008). In a study examining the efficacy of iron supplementation, Baumgartner et al. (2013) report that South African children with high BMI-for-age-z-scores have a greater risk for remaining iron-deficient after iron supplementation for 8.5 months when compared to children with low BMI-for-age-z-scores. These seemingly contradictory findings may be due to a complex set of environmental and individual variables that include differences in disease exposure and immune activation caused by specific economic and cultural contexts. Additionally, while BMI has traditionally been used as a proxy for body fat (Albrecht et al. 2014), visceral adiposity or waist circumference may provide greater insight into the relationship between adiposity and anemia due to differences in in the production of inflammatory cytokines by fat tissue.

INTESTINAL MICROBIOME

The intestinal microbiome is especially important to investigate when exploring anemia because of its documented association with both under-nutrition (Gleason and Scrimshaw 2007) and over-nutrition (Aberli et al. 2011; Eftekhari et al. 2009). Recent advances in technology have allowed for more in-depth investigations of microbes living in or on human hosts. This collection of organisms and their genomes is known as the human microbiome (Qin et al. 2010). Changes in microbial communities have been implicated in the cause of several chronic conditions, specifically with the trillions of micro-organisms living in the human gut (Wu et al. 2013; Larsen et al. 2010; Manichanh et al. 2006). Evidence has also demonstrated links between taxonomic composition of the gut microbiota and malnutrition.

Results of these studies suggest that gut microbial composition is a potential cause of as well as protect against weight gain. Turnbaugh et al. (2009) report that phylum-level differences, decreased bacterial diversity, and altered representations of bacterial genes and metabolic pathways are associated with obesity. In a prospective study, Kalliomaki et al. (2008) found that changes in the gut microbiota preceded weight gain in a cohort of Finnish children. In addition to being implicated in contributing to overweight and obesity, the microbiome has also been linked to undernourishment. While the primary etiology of under-nutrition is associated with prolonged negative energy balance due to nutrient depravation and deficiencies, studies demonstrate a distinct relationship between undernourished children and their gut microbiome. Subramanian et al. (2014) demonstrated that severe-acute malnutrition is associated with significant relative microbiota immaturity in Bangladeshi children. Similarly, Smith et al. (2013) found the structure and genetic contents of the intestinal microbiome were different between Malawian twin pairs that were discordant for Kwashiorkor. In both of these studies, a dominance of Proteobacteria and a low diversity of gut microbiota were observed in undernourished children and were attributed to the lack of child response to nutritional interventions.

The intestinal microbiome may also play a key role in iron absorption through microbial metabolism (Petry et al. 2012; Tako et al. 2008) and alterations in intestinal pH level (Salovaara et al. 2003). However, some micro-organisms do not establish a symbiotic relationship with humans when iron is introduced to the gut. While iron is an essential metal for humans it is also vital for the growth and proliferation of many pathogenic bacteria.

Generally, iron in the human body is bound to proteins which limits iron availability to potential infective agents. During infection, the innate immune response sharply reduces iron absorption and sequesters iron to further decrease bioavailability (Oppenheimer 2001). However, this immune defense sequence leaves iron in the gut. In the duodenum and upper jejunum, bacteria compete for unabsorbed dietary iron because the availability and ability to acquire this vital metal is essential to bacterial colonization, and, for most enteric gram-negative bacteria, also plays an essential role in virulence (Naikare et al. 2006). The immune response against pathogenic bacteria is limited in the intestine as there is no system for the sequestration of iron in the gut lumen (Andrews et al. 2003). While recent studies have not revealed significant differences in phylogenetic diversity between anemic and iron-replete infants and children, there are documented differences in taxa abundance. Jaeggi et al (2015) found that anemic Kenyan infants had lower abundances of *Prevotell*a and higher abundances of *Actinomycetales* and *Streptococcus*. In a study with Kenyan children, better iron status was shown to predict lower amounts of *Escherichia coli* (Paganini et al. 2016).

Despite fortification being an effective strategy to reduce anemia rates (Zimmermann and Hurrell 2007; Baltussen et al. 2004), iron supplementation may lead to a greater concentration of unabsorbed iron in the gut (Kortman et al. 2014). This creates an environment that favors increased colonization of pathogenic bacteria over barrier or protective bacteria, thus generating gut microbiota disequilibrium and increasing morbidity. Recent studies demonstrate the negative effects of iron fortification on childhood morbidity rates and gut microbiota composition. Research in Côte d'Ivoire found that children receiving iron fortified wheat flour had an increase in the number of enterobacteria and iron deficient children had unfavorable ratios of fecal enterobacteria to bifidobacteria and lactobacilli at baseline, which only increased with iron fortification (Zimmermann et al. 2010). Jaeggi et al. (2015) demonstrated that the provision of micronutrient powders (MNP) to weaning infants increased the prevalence of Enterobacteria and *Clostridium* and the enterobacteria/bifidobacteria ratio in Kenya. This work provides valuable insight on the gut microbiome as a pathway between iron fortification and morbidity, however its geographic scope is limited to sub-Saharan Africa, ignoring other areas of the world suffering from high anemia prevalence.

DEVELOPMENTAL MICRONICHE

Factors that affect child pathogen exposure, nutritional status, and the composition of intestinal microbiota involve the complex interplay of political, ecological, social, and biological factors. The concept of the developmental microniche (Super and Harkness 2002; Worthman 1994) is a useful model for exploring the relationship between socioecological context and health. The niche is defined as the variable individual context of each child, and includes the social as well as physical settings in which each child develops (Harkness and Super 1986; Worthman 2010). The niche provides a level of analysis and explanation between the more proximate (physiological) and distal (social) aspects of biology, and a framework to organize and explore relationships between children's biology and their socio-ecological context (e.g., Brewis 2003). The developmental microniche, therefore, serves as a useful guide for methods and analyses to explore how individual anemia status and efficacy of iron supplementation can be linked to national campaigns.

PERU

Peru provides an important setting to study anemia and iron supplementation from evolutionary medicine and LHT perspectives within a dual burden context. This Latin American nation suffers from high anemia rates, making it similar to many sub-Saharan African countries, where anemia rates tend to be highest (Alcázar 2013). The WHO (2009) has categorized Peru as having "severe" anemia prevalence and estimates that about half of all pre-school age children, pregnant women, and non-pregnant women of reproductive age suffer from anemia (Figure 1.2). The prevalence of anemia in children under five is higher than the prevalence of malnutrition and has remained constant despite decreases in both stunting and poverty rates (Marini et al. 2017). The high rates of anemia in Peru have received considerable governmental and non-governmental attention and several initiatives have aimed to reduce levels of anemia among children five years and younger, including the most recent campaign *Plan Nacional para la Reducción de la Anemia* 2017-2021 (National Plan to Reduce Anemia) (Figure 1.3). Despite these repeated interventions for reducing anemia and malnutrition more broadly, anemia continues to represent a distinct challenge, even for those living near large cities who have greater access to urban infrastructure.

Lima is the largest city in Peru, located on the Peruvian coastal plain (Figure 1.4). The capital city is the political and financial center of the country. This results in substantial internal migration to Lima, contributing to an annual urban growth rate of 1.57% (INEI 2013). One outcome of Lima's growing population is the expansion of peri-urban communities. These communities are characterized by informal or poor-quality housing, unhealthy living conditions, and poverty, resulting in greater exposure to risk factors and negative health outcomes.

San Juan de Lurigancho a peri-urban district in the north-east quadrant of Lima, has been the site of numerous anemia interventions to date (Figure 1.5). In 2013, 35.7% of children under five living in this district were diagnosed with anemia; in 2014, despite the efforts of the Ministry of Health and community-based organizations, that percentage had *increased* to 41.9% (Ministerio de Salud 2015). San Juan de Lurigancho therefore provides an ideal context to investigate anemia and iron supplementation due to the community's high rates of anemia, the lack of success among programs that seek to combat it, and the expressed anxiety about anemia from community members.

CHAPTER OUTLINES

This dissertation explores several hypothesized pathways for childhood anemia and a lack of response to iron supplementation in a peri-urban, Peruvian context. The following chapter provides an ethnographic description of San Juan de Lurigancho and its inhabitants to illustrate the day-to-day experiences, encounters, and challenges of residents as well as the complexities of collecting data within this community. The events described in this chapter were chosen because of their connection to the neighborhood, health, and nutrition. They take place in several locations throughout the community, including the *tren electrico* (electric train) that connects San Juan de Lurigancho to the city center, the steep, dusty roads that wind through the neighborhood, and the homes of several participants.

Chapter 3 describes the methods used throughout this project. This chapter begins with a description of the study design, followed by the protocol for data collection. I highlight important study variables and describe the laboratory and statistical analysis methods used to discover the results reported in my three dissertation articles, presented here as Chapters 4, 5, and 6.

The first dissertation article is presented in Chapter 4. This chapter aims to identify general socio-demographic features that may explain the particularly high prevalence of anemia in a peri-urban Peruvian population and the lack of success of national anemia programs. As previously noted in this introduction, despite repeated governmental and nongovernmental interventions, anemia remains a widespread public health concern in Peru. Data come from children, aged 2-5 years (n=102) living in San Juan de Lurigancho. Predictors of anemia and response to iron supplementation were explored at the individual, maternal, household, and environmental level using logistic regression models adjusted for siblings. Half of the children in this sample were anemic and 50% of the anemic children responded to iron supplementation. Lower weight-for-age z-scores and the winter season were strong predictors of child anemia status and non-response to iron supplementation. Living with paternal grandparents was protective against anemia and elevated CRP at the time of the final interview was associated with a lack of response to iron supplementation. The findings of this study allow some recognition of the association between children's anemia status and socio-ecological context, highlighting the importance of examining anemia across diverse contexts to better understand the factors driving this endemic problem

Chapter 5 investigates the relationship between high immune activation and lack of response to iron supplementation after one month of treatment and explores variation in body fat stores as a potential moderator between immune function and response to treatment. Peruvians are experiencing rapid dietary and lifestyle changes, obesity has emerged as a threat equal to illnesses associated with undernutrition, an example of the phenomenon known as the 'dual burden of disease'. A common manifestation of the dual burden in individuals is the co-occurrence of overweight and anemia. Despite recent initiatives introduced by the Peruvian

Ministry of Health and community-based organizations to address the coincidence of obesity and anemia, rates continue to be public health concerns. Data come from children, aged 2-5 years (n=50) from a peri-urban community in Lima, Peru. Multivariate logistic regression models were used to explore the associations between anemia, markers of immune activation (CRP and reported morbidity symptoms), and measures of body fat (waist-to-height ratios, triceps skinfold thickness, and body mass index). I found that high immune activation is associated with a lack of response to iron supplementation after one month of treatment and that body fat moderates the association between immune function and response to treatment. Different adiposity measures provide variation in the probability of anemic children responding to iron supplementation treatment. While I expected a reduced probability of response to iron supplementation in children with high immune activation and high body fat, this pattern did not remain with higher BMI z-scores. Through the inclusion of markers of immune activation and several measures of adiposity, these results further our understanding of the relationship between inflammation and anemia in children in areas experiencing the dual burden of disease.

Chapter 6 uses an evolutionary medicine perspective to explore the role of increased iron availability via iron supplementation and intestinal microbiota diversity on recovery from childhood anemia in Peruvian pre-school-aged children. I test the gut microbiome as a hypothesized pathway that may link iron supplementation and child recovery from anemia. This study asks: Does one month of treatment with iron supplementation significantly alter the diversity and composition of the gut microbiome? Do baseline and post-treatment gut microbiota diversity and predominant taxa differ by response and non-response to iron supplementation? Do these differences persist after controlling for relevant pathogenic exposures? I tested for differences in alpha and beta diversity as well as taxa abundance between pre and post supplementation samples, and response to iron supplementation using QIIME and Stata 13. For taxa showing evidence of difference in bivariate testing (p-value<0.25), I ran multivariate regression models to assess whether the abundance of taxa differed by timing and response when controlling for pathogenic exposures. I did not find differences in diversity between pre- and post-supplementation samples and within baseline and post-treatment samples. While there were a number of differences observed in relative abundance between pre- and post-supplementation samples and responders and non-responders at baseline, few of these differences were greater than 1% relative abundance. In models controlling for pathogenic exposures, response to iron supplementation was associated with a lower abundance of Proteobacteria (p-value = 0.06), Enterobacteriales (p-value = 0.02), and Lactobacillales (p-value = 0.01) in post-supplementation stool samples. Through the use of an evolutionary medicine perspective as well as the inclusion of developmental microniche methods to create pathogenic exposure variables, this study provides evidence for the gut microbiome as a pathway that links iron supplementation and child recovery from anemia. These findings suggest that investigating pathogen exposure and microbial health is important to better understand the impact of iron fortification on child health and development.

In the final chapter, I synthesize the results and significance of the three articles. I also highlight the strengths and weaknesses of the project and outline its potential broader impacts and ideas for future, related, research.

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FIGURES

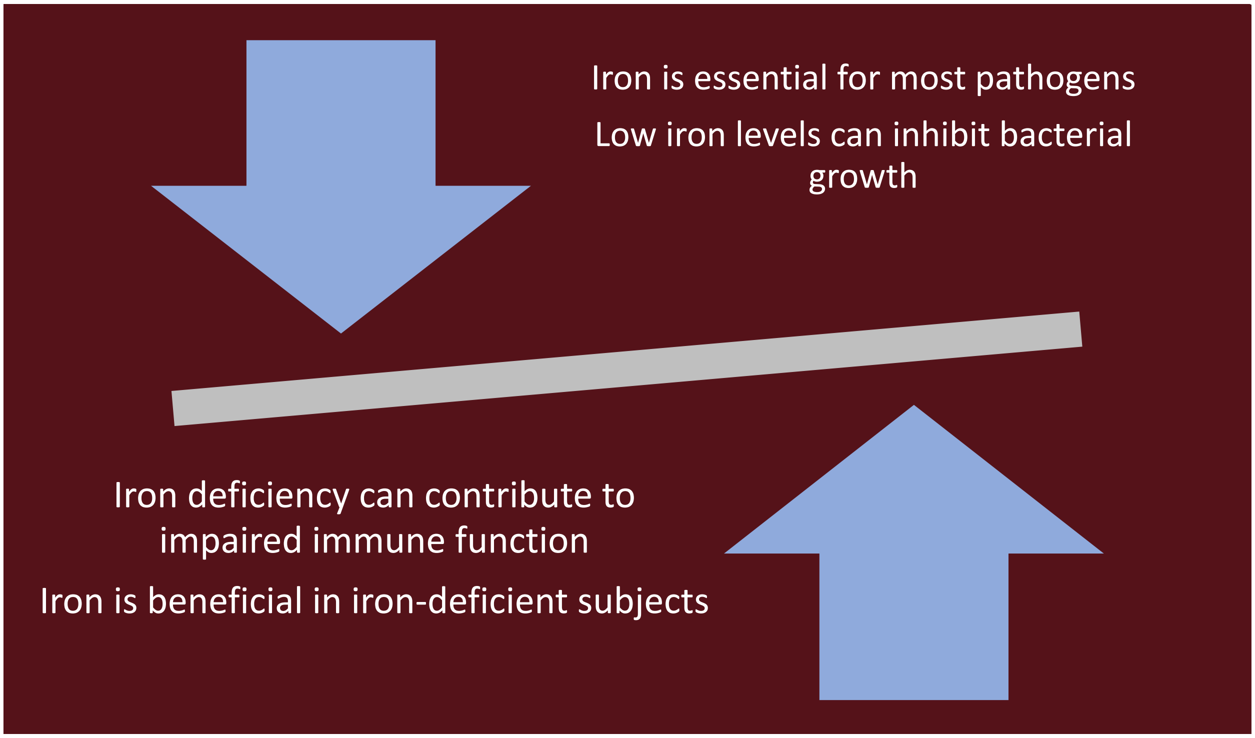

FIGURE 1.1: Conceptual model of iron level from an evolutionary perspective

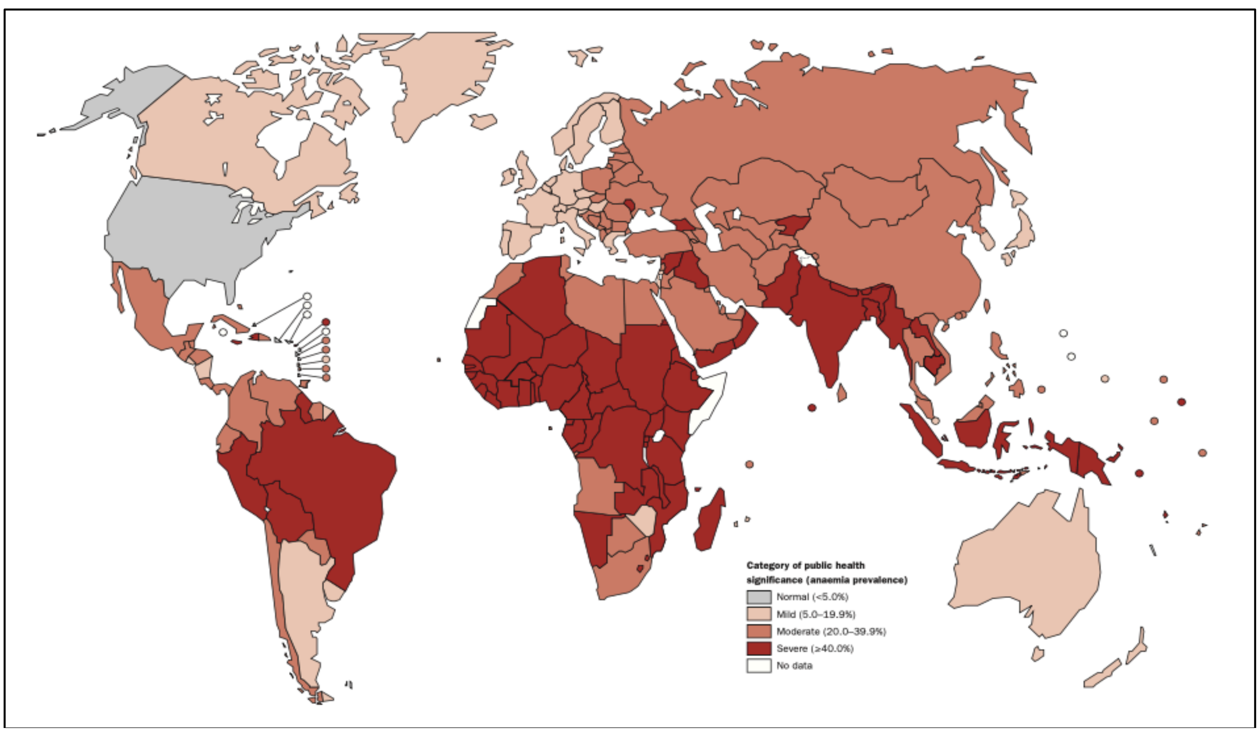

FIGURE 1.4: Anemia as a public health problem by country: Preschool-age children (WHO 2008)

FIGURE 1.5: Marketing materials for the Plan Nacional para la Reducción de la Anemia 2017-2021 (minsa.gob.pe)

FIGURE 1.2: Map of Peru

FIGURE 1.3: Map of Lima, Peru

CHAPTER 2. SAN JUAN DE LURIGANCHO

The screech of the Metro de Lima on the platform above me signals to the crowd of people in the Ayacucho station to hurry. Men and women in suits carrying briefcases and dark backpacks pick up their pace and the clatter of their shoes on the concrete steps encourages me to run as well. As the crowd and I reach the top, a wave of people pushes toward the doors of the nearest green and white train car. The metro is already crowded with professionals heading towards the historic center of Lima, and everyone pushes their way into the train before the doors close. Linea 1 of the *tren electrico* (electric train) links the southern district of Villa el Salvador and the northern district of San Juan de Lurigancho, carrying thousands of passengers across Lima each day.

While the packed train is uncomfortable, I already know that riding the elevated metro to San Juan de Lurigancho is preferable to facing the heavy traffic that routinely clogs the roads throughout the bustling capital. The crowd on the train thins slightly with each passing station and shrinks dramatically after we pass through the three stops within the center of Lima, where the majority of middle-class professionals work. Once we are through the city center, the view out of the window shifts from tall colonial buildings to smaller contemporary houses as the metro winds its way to the outskirts of the city. Soon after this change in scenery, I spot the hills around northern Lima, covered with colorful but unfinished cinderblock homes.

By the time the train pulls into Santa Rosa, the second to last station on this metro line, there are only four people left in the train car. When the train doors open, I am greeted by the cacophonous sounds of car horns and shouts from amidst the traffic below. I pull out my phone and notice a series of texts from Gisella Barbagelatta, the field coordinator for this project, "*¡No te olvides de esperar!"* (Don't forget to wait!) *"¡Ten cuidado!*" (Be careful!). These messages are reminders to wait on the train platform until I spot the dark blue van that belongs to the *Instituto de Investigación Nutricional* (IIN). I know that Gisella worries that I am an obvious target for theft, or worse, if I wait among the crowds below. While I think these concerns are exaggerated, I wait until I see the van park on the other side of the road. The stairs are empty on the left side of the station but there is a line of people waiting on the opposite platform that stretches down the stairs and past the turnstiles towards the busy street. The metro is one of the fastest ways for people living in San Juan de Lurigancho to reach the wealthier communities in Lima, where many work as domestic laborers and municipal workers.

I dodge several moto-taxis, cars, and buses to reach the vehicle, Gisella and Ivan, the driver, tease me as I climb into the back seat. They tell me that my height and curly hair make me stand out from the other people weaving in and out of traffic. We laugh together at my inability to blend in, but stop short when several people stumble out of a large purple bus, grappling, throwing punches, and shouting. One of the men had snatched a woman's wallet and tried to flee the scene when two older men intervened. Ivan shakes his head and starts the van, he makes eye contact with me in the rearview mirror and says "*y ahora vamos a trabajar"* (and now, we go to work).

THE HEALTH CENTER

This was the first morning of my first day working in San Juan de Lurigancho. For nine months, I commuted to the health center by train every weekday. The pediatricians, nurses, and researchers working at the center were funded by the IIN and they carried out a number of health programs and studies in accordance with the goals of the Institute and its funding agencies. Like many of buildings in San Juan de Lurigancho, the health center was made from concrete and brick but it stood out from its neighbors because of its bright green paint and rust-colored trim. The right side of the center was for health care and consultations for adolescents and adults while the left, where my office was located, was home to the pediatrician's office and a small laboratory. Blocks, musical instruments, and pink plastic tea cups were often scattered across the tiled floor in the foyer, which served as the waiting room for visiting mothers and children. Juanita, the health center's receptionist, would collect the family's information and then measure and note the child's height and weight before calling down the hall to Dr. Theo, the resident pediatrician.

Juanita was a jovial older woman who loved holding infants and would sing to the children receiving vaccinations in order to distract them from the pain while wiping their tears. During quiet days at the center, Juanita and Ivan could be found sitting in the lobby watching *telenovelas* (soap operas) or national soccer games and sharing a liter bottle of Inca Cola, a bright yellow soft drink common throughout the Andes that tastes like bubblegum. Ivan not only drove the center's health workers around the community, he also helped Dr. Theo with car maintenance. The Doctor's dark blue car had a reputation for breaking down and every employee at the center had a story—including me, eventually—about a harrowing adventure that unfolded when the car suddenly stopped working. While Dr. Theo complained endlessly about his lack of trust in the vehicle, he carefully wiped the dust off of the car doors and windows each morning after arriving to the center.

Gisella, who would be my near constant companion during data collection, grew up in San Juan de Lurigancho and was immensely proud of her two daughters, both of whom were university students. Stout and rosy cheeked, she was the heart of the office never forgetting a birthday and quick to arrange celebrations for any major event in her coworkers' lives. Gisella's office was upstairs away from the other center workers. She liked the location because it faced the front of the center and had large windows. Being able to look out over the community allowed Gisella to spot caregivers—almost always mothers—and children on their way to the center well before they rang the bell at the gated front door.

Mothers in this community purchased food at the local market, choosing its maze of stalls over travelling to the relatively new supermarket chains farther away. Many worked outside the home as vendors selling food or clothing, as cooks in restaurants, or as maids in wealthier households closer to the city center. Their occupational status was fluid and changed throughout the year. Mothers often worked multiple jobs and longer hours before the start of school in order to secure the funds necessary to pay for uniforms, school supplies, and school fees for their children. Homes were often multi-generational, with nuclear families occupying different floors and sharing the kitchen area.

Mothers typically brought their children to the center with multiple health concerns and it seemed that the higher up the hill they lived, the more numerous the problems that brought them to the center. Rashes, sore throats, and persistent coughs were common ailments. When children with cases of severe conjunctivitis (pink eye) left the center with their mothers after receiving a prescription note, Juanita would quietly pick up the toys they had been playing with and wash them to reduce the chance of spreading the bacteria. Mothers almost always filled any prescriptions their children received at the health clinic across the street, which had a red sign hanging in the window, declaring "*Queremos niños sin anemia*" (We want children without anemia).

COMMUNITY HISTORY AND STUDY DESIGN

San Juan de Lurigancho is one of the many peri-urban communities around Lima. These communities are the combined result of policies that favored highland landlords over rural peasants prior to Peru's agricultural reforms and of the promise of higher wages available in the expanding capital. The growth and number of these unplanned communities increased dramatically in the 1980s and early 1990s when violence in the highlands perpetrated by *Sendero Luminoso* (Shining Path) and the state's brutal counter-insurgency forces drove another influx of migrants to Lima (Theidon 2010, Seligmann 2004). During this period, over 600,000 peasants emigrated from the highlands (Theidon 2010). Substantial internal migration to Lima still occurs, leading to an annual growth rate of 1.57% (INEI 2013). While urbanization is linked with higher levels of education, literacy, and economic productivity (United Nations 2014), rapid urbanization often fails to sustain healthy populations due to a greater demand on water reserves, sewage management systems, and health services as well as a higher population density.

Peri-urban communities often serve as "laboratories" where the Ministry of Health initiates planned health interventions before scaling up these programs to the national level. Two such programs are the Initiative against Chronic Infant Malnutrition and *Mesa de Concertacion de Lucha contra la Pobreza* (Poverty Reduction Roundtable), which helped reduce the rate of chronic childhood undernutrition from 28 to 13% between 2006 and 2016 (Marini et al. 2017). These programs typically provide supplementation through syrups and micronutrient powders or additional food items as the Peruvian diet is typically low in iron and other nutrients (Creed-Kanashiro et al. 2003). More than half the children who enrolled in my study were actively participating in a child food program, the most common being *Vaso de Leche* (Glass of Milk), the largest social assistance program in Peru (Parodi 2000), which distributes milk to expectant mothers and children under seven[1]. National programs also provide funding for school snacks. In

[1] The national milk program (Law 24059) was created in January 1985 in response to a women's march in Lima (about 25,000 demonstrators) demanding that all children should have the legal right to a glass of milk each day in December 1984 (Copestake 2008).

San Juan de Lurigancho, the pre-schools typically offered a hard-boiled egg and bread to students each day, however, most caregivers also provided food for their child to take to school. These snacks were typically pre-packaged cereals or pre-made sandwiches and juices purchased from local vendors with push carts or kiosks that they operated out of their homes.

Most homes in my study had an in-house water connection, however, every mother discussed boiling and then storing water in plastic containers or pitchers for consumption at a later time. This practice is common throughout Lima, because despite the use of high levels of chlorine and filter systems, water becomes re-contaminated when introduced to the municipal water supply. Keeping water clean, however, can be a challenge as the microbiological quality of water can deteriorate during home storage, potentially resulting in fecal contamination (Oswald et al. 2007).

Nutrient deficiencies were addressed in the aforementioned programs, through supplementation and fortification strategies. However, despite the success of these programs in reducing malnutrition in children, anemia rates remained high. Due to this persistence, anemia has received considerable government and non-government attention. Several initiatives have incorporated plans to reduce levels of anemia, specifically among children five years and younger. The *Plan Nacional para la Reducción de la Desnutrición Crónica y la Anemia Infantil* (National Plan for the Reduction of Chronic Malnutrition and child Anemia) was adopted in 2011 by the government under Peruvian President Ollanta Humala. President Pedro Pablo Kuczynski Godard (also known by his initials, PPK) continued his predecessor's focus on anemia and created the most recent campaign, *Plan Nacional para la Reducción de la Anemia* 2017-2021 (National Plan to Reduce Anemia) which brought together the Ministry of Health and regional and local governments, as well as community-based organizations and citizens to eradicate anemia as a public health problem[2].

These programs aimed to reduce anemia in children under five years of age by distributing preventative and therapeutic ferrous sulfate and multi-micronutrients and were modeled after successful national-level plans to reduce child undernutrition. Non-governmental organizations (NGOs) associated with current and previous anemia and malnutrition reduction efforts continue to partner with community organizers to spread information about the importance of iron and the debilitating effects of anemia on child brain development in local community centers and kitchens as well as via school programs.

These programs have led to an evident increased awareness of anemia. During preliminary fieldwork, mothers voiced a preoccupation about the effects of anemia on their child's cognitive and physical development. As a bio-cultural anthropologist, I have always felt a strong ethical commitment to design studies that reflect the interests of those living in the communities where I work and to conduct research with value beyond academia. Because of this, I developed this dissertation project with the mothers' concerns about anemia in mind and designed a project to examine hypothesized predictors of child anemia status and response to iron supplementation, including potential socioeconomic and biological pathways that influence hemoglobin levels.

While collecting data I discovered that almost all of the mothers that participated in my study had some knowledge of the illness and that most of these women learned about anemia through programs at school or had direct experience with anemia when they (or someone they

[2] Pedro Pablo Kuczynski Godard announced his resignation on March 28, 2018 after 20 months of serving as the Peruvian President due to an ongoing investigation into corruption and money laundering within his administration. While fieldwork was being conducted the National Plan to Reduce Anemia continued despite a new president and cabinet, but as of June 2019 the website dedicated to the program no longer exists.

were close to) were pregnant. Despite this awareness, anemia rates remained high: half of the 102 children in my sample were anemic. I monitored anemic children over the course of their prescribed anemia treatment (one month). During the four-week period, I spent time with the child and their mother in both the health center and in their household to learn about how caregivers and children experienced both anemia and its treatment.

ANEMIA IN SAN JAUN DE LURIGANCHO

Caregivers' reactions to a child's anemia status at the initial interview ranged from unfazed to disbelief. One mother, after learning that her four-year-old daughter was anemic declared "*¡Pero ella es gordita! ¿Como puede tener anemia*?" (But she is chubby! How can she have anemia?). Another mother brought her twins to be tested for anemia and was surprised to find out that the larger of the two was anemic while the smaller one was not. In this study, mothers tended to associate size and potential anemia status, often seeking treatment because they were worried about their child's small size or lack of appetite. The assumption that body size was predictive of health status often led to surprise when caretakers learned about their child's anemia status.

Samuel[3], a four-year-old boy enrolled in my study, lived with his mother, Carmen, and three older brothers in a second-story apartment with bright pink walls that reminded me of Pepto-Bismol. Giselle and I were visiting the house for our second home visit and Samuel was pleased to see me and asked for the crayons and paper I brought to each interview within seconds of my arrival. While he set up the art supplies on makeshift furniture made from construction materials and plywood, Carmen complained about how difficult it was to get to the train station and travel to Miraflores for her job and how much she was enjoying her day off. During the

[3] This, along with all other names of study participants and their caretakers, is a pseudonym.

interview she laughed easily and often, revealing a missing bottom tooth. When I asked her about giving Samuel the ferrous-sulfate syrup during the previous two weeks, she threw her hands up in the air, admitting she almost always forgot in the morning because that time was "*ocupado*" (busy).

"*Porque tengo cuatro hijos que necesitan ir a la escuela y necesito ir a trabajar*" (because I have four children that need to go to school and I need to go to work), she explained. "*Si tuviera un hijo nunca me olvidaría de darle*" (If I had [only] one child, I would never forget to give it), she continued, reminding Gisela and I of the stress inherent in mothers' daily responsibilities, something common in nearly every human society. Her honest appraisal of her limitations also suggested to me that studies of iron supplementation need to consider the number of children at home—not just children with anemia.

Farther up the hill, Apryl, another mother, blamed a different set of distractions for her lack of adherence with the study protocol. Gisella had insisted we visit Diego, Apryl's two-year old son, in the mornings because the narrow staircase that lead visitors to Diego's home was often occupied by alcoholics and drug dealers in the afternoons. As we approached the cardboard and plywood walls of their improvised shelter, Diego's father greeted us by waving his hand and taking a drink out of the wine bottle in his right hand. His eyes were red and surrounded by a white discharge, reminding me of the child diagnosed with pink eye at the health center earlier that month.

Apryl opened the door revealing an earthen floor dappled with light filtered through the unfinished corrugated roof above. She was busy cooking rice and answered our questions quickly and without much patience. Diego had no interest in the crayons and paper I offered him and instead cried and pulled at his ponytail. The crying did not cease, which distracted Gisella and I so much that eventually I stopped the interview so Gisella could ask Apryl what was wrong with her child. Apryl did not answer and instead took Diego's sweatshirt off, revealing bright red blood on the collar and a mix of scabs and open sores on his arms and neck. Gisella recognized the symptoms of skin mites immediately and began telling Apryl that she needed to come to the health center and get a prescription from the pediatrician for treatment later that day. When Gisella and I left, she was unusually quiet. As we reached the bottom of the staircase and turned right to return to the health center via the dirt road with the fewest stray dogs, Gisella took a deep breath and commented that *"…la adherencia al tratamiento de la anemia no debería ser la principal preocupación de Apryl…"* (adherence to anemia treatment should not be Apryl's main concern). Gisella and I understood that while chronic anemia has detrimental effects on child growth and development, acute health problems often must take precedent over treatment for chronic conditions.

Maria, aged three, arrived at the final interview with a large bruise on her forehead. The bruise didn't seem to affect her energy and as she skipped up and down the halls of the center swinging a stuffed purple bunny, her mother, Anna, described the incident. Maria did not like the taste of the syrup and whenever she saw her mother take the bottle out of the cupboard, Maria would sprint in the opposite direction. The morning of the final interview, Maria turned to run away but tripped and fell down the stairs. Child reactions to iron sulfate ranged from liking the syrup and asking for more to running away or fighting with the caregiver who was trying to get the child to take their medicine. Several children described the taste of the syrup as "*feo*" (disgusting) and did not want to eat it, Maria belonged in this group.

Sumaq and his mother Isabella lived a short block away from the health center. Their home had concrete walls and the living room had a large bright orange furniture set arranged around a flat screen television. Sumaq insisted on using his own colored pencils to draw during the interview and gave me a picture of a man with six arms before I left. Sumaq had long black hair and a wide flat face that reminded me of the Inka portraits I had seen in some of Lima's museums. Isabella was the only mother who identified herself and her son as Quechua, and the connection she had with her ancestry was reflected in the indigenous names she had given her children. She applied her knowledge of Andean history when I asked her whether giving iron supplementation to Sumaq was easy or hard. Isabella admitted to becoming so frustrated with her four-year old son's resistance to opening his mouth that she threatened to make him "*como Túpac Amaru*." She was, of course, referring to Túpac Amaru II, a leader of an indigenous uprising against the Spanish in the late 18th century. His torture and death included being drawn, quartered, and beheaded in Cuzco's main plaza (Chamber and Chasteen 2010). While this seemed like a shockingly violent threat for a mother to make to her son, Sumaq was unphased and continued to refuse to take the iron-fortified syrup. As anyone who has worked with children—or has children of their own—knows, personality plays a significant role in how easy it is to get a child to take medicine. Sumaq's mother was not the only caretaker that talked about needing to use "*fuerza*" (force) to get their child to take the spoonful of syrup two times each day, though she was the only one to invoke a Quechua martyr in her efforts. Her story highlights the importance of child agency, personality, and taste preferences in adherence to treatment protocols.

Even with high compliance, only half of the anemic children responded to one month of iron supplementation treatment. Adriana brought her two children Pablo, aged 3, and Gabriella, aged 2, to the health center for the final interview. Adriana seemed invested in her children's recovery, at each interview she would ask questions about the treatment protocol to ensure she understood correctly and expressed concern about not having enough of the treatment for the full month. At the last interview, Adriana wanted to let us know that she never ran out of the syrup and that she had never missed a dose for either of her children. When we tested her son Pablo's hemoglobin, she was thrilled to learn that he was no longer anemic and threw her arms up in the air to celebrate with him. Gabriella's hemoglobin decreased slightly at the final interview and upon learning this, the mother's posture changed and her smile disappeared. "*¿Qué significa esto?¿Qué puedo hacer de manera diferente? Hice todo lo que pude*." (What does this mean? What can I do differently? I did everything I could). A number of mother's in this study acted similarly, they celebrated if their child recovered but became frustrated if they did not.

REFLECTIONS

The ups and downs of data collection reminded me of a conversation I had with a mother who declined to participate in my study. Gisella and I had wandered up the dirt road leading away from the clinic and towards the Santa Rosa station for the Metro de Lima and knocked on several doors to enroll potential participants in my study. When we explained the protocol and the benefits of diagnosing and treating anemia, to this particular woman, she leaned against the doorway holding a basket of laundry, shook her head, and responded "*no, no necesito otra preocupación*" (No, I don't need another worry). I was discouraged and concerned, how could this woman not want to know about her child's health status?

This was not the only time I was irritated by the behaviors and responses of others during my time in San Juan de Lurigancho. As I continued to interview mothers, play with children, and walk along the community's dusty roads, however, my feelings shifted. I could not be disappointed in the people of San Juan de Lurigancho. Instead, I found myself frustrated with the overwhelming structural inequalities that continued to shape the community's social and physical landscape and limit its residents' upward mobility, often despite their very real efforts.

Fieldwork changed me in ways that I cannot fully articulate or even always understand. I was forced to acknowledge my weaknesses and encouraged to discover new strengths. While I learned that every case is unique, in order to adequately reduce childhood anemia, I believe we must shift our attention from individual behaviors and address the conditions in which mothers and children live, work, and play.

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FIGURES

FIGURE 2.1: The health center in San Juan de Lurigancho

FIGURE 2.2: Overlooking the neighborhood soccer field and hills surrounding the area

FIGURE 2.3: A moto-taxi parked on a narrow street

FIGURE 2.4: A neighborhood staircase and a friendlier street dog

FIGURE 2.5: On a hill overlooking the expanding peri-urban community

CHAPTER 3. STUDY DESIGN, DATA COLLECTION, AND MEASURES

Between November 2017 and July 2018, I collected qualitative and quantitative data from caregivers and anemic preschool-age children living in three neighborhoods within the San Juan de Lurigancho district of Lima, Peru. Research in this community was conducted in collaboration with researchers at the *Instituto de Investigación Nutricional* (IIN), a private, non-profit institution dedicated to interdisciplinary research on health and nutrition in Peru. This study received both University of North Carolina at Chapel Hill IRB and IIN Ethics Board approval for this project. Parental consent and child assent were obtained prior to enrollment in the study.

STUDY DESIGN AND DATA COLLECTION

Recruitment and Initial Interview

The study field coordinator, Gisella Barbagelatta, and I recruited 102 caregivers (typically mothers) and their children (aged 2-5 years) through door-to-door and "snowball" sampling. All maternal-child dyads participated in an initial interview at the local health center that included hemoglobin (Hb) assessment. The initial interview took place in a small private room at the center that included an extensive demographic survey, morbidity symptom report, 24-hour dietary recall and anthropometric measurements.

At the conclusion of the interview, the child's capillary Hb was measured to test for anemia via a minimally invasive finger prick and a portable photometer Hemocue machine (Hemocue Hb 201+, HemoCue America, California). The child's finger was cleaned with alcohol, and a sterile disposable microlancet was used to deliver a controlled puncture. Whole blood was placed directly onto the Hemocue cuvette and inserted into the Hemocue machine.

The Hb measurement was conducted within 30 seconds of the finger prick and was used to determine if the child was or was not anemic using the World Health Organization's definitions of iron status (WHO 2009). At least one drop of blood was collected from participants after the finger prick for Hb measurement on standardized filter paper (Whatman #903. Middlesex, UK) for analysis of C-reactive protein (CRP), a direct measure of immune activation.

Half of the children participating in the initial interview were not anemic (n=51) and were excluded from further interviews. If the child was anemic, the participant visited the health center pediatrician and was prescribed ferrous-sulfate syrup, a treatment with a high concentration of iron. Treatment included consuming a tablespoon of ferrous-sulfate syrup two times a day, once in the morning and once in the afternoon at least 30 minutes before a meal for 28 days. This treatment followed the anemia intervention guidelines provided by the Peruvian Ministry of Health (Ministerio de Salud 2015). The maternal-child dyad participated in three additional interviews over the course of one month, the time period during which the child should respond to the treatment.

First Home Visit

The first home visit occurred 24 hours after the initial interview and took place in participant's homes in a room of the caregivers' choosing (most often the kitchen or living room). The visit included a qualitative interview which had open-ended questions about child personality and physical activity, community environment, and health. The mother also responded to a hygiene questionnaire and I used an observation checklist and free-form notes to document child activities and household environment during the interview. At the end of the interview, I would pick up the child stool sample the mother had collected and saved in her freezer while Gisella noted the date, time, and location of sample collection.

Second Home Visit

Two weeks after the initial interview, Gisella and I would conduct the second home visit. This interview included a morbidity symptom checklist, 24-hour dietary recall, open-ended questions about adherence to treatment, a food insecurity checklist, dietary diversity questionnaire and a second Hb measure. I also used the observation checklist and free-form notes to document child activities and household environment during the visit. Considerable time was spent in ethnographic observation during the home visits and would typically include playing with the child, eating meals with the family, and walking the child to school.

Final Interview

The final interview took place in a private room at the local health center after four weeks of treatment. This interview included a morbidity symptom report, 24-hour dietary recall, open-ended questions about compliance to the treatment protocol, a second stool sample, and a final Hb test using Hemocue Hb 201+ to assess the child's response to treatment. Additional spots of blood were collected from participants after the finger prick to assess CRP levels after one month of treatment.

KEY STUDY VARIABLES

Anemia and Response to Iron Supplementation

In our study, we used Hb as an objective measure of anemia status. Hb concentration is the most common hematological assessment method used to measure anemia (Chaparro and Suchdev 2019). In Peru, recent initiatives by the Ministry of Health use Hb to assess anemia status in national-level programs at health centers and in schools, incorporating methods used by these initiatives allows for comparison between reported prevalence and rates of anemia in this sample. Additionally, measuring Hb is inexpensive and easy to measure with field-friendly testing. Hb concentration does lack specificity for establishing nutritional anemias, such as iron status (Balarajan et al. 2011).

In the following analyses, anemia is used as a dichotomous variable (iron replete ≥11.0g/dL and anemic <11.0g/dL). These categories are based off of WHO recommendations for Hb (2011), which have not changed since 1968 but continue to be used in a number of studies and publications. Response to iron supplementation is also used as dichotomous variable (responder and non-responder). Children who became not anemic (≥11.0 g/dL) after 1 month of treatment were labeled as responders while children whose Hb level remained below 11.0 g/dL at the final test were categorized as non-responders.

Other studies investigating anemia from an evolutionary perspective use specific measures for iron status, such as serum transferrin receptor (sTfR) and zinc protoporphyrin to heme ratio (Hadley and DeCaro 2015; Wander et al. 2009). Analyses of these measures are typically conducted with plasma extracted from venipuncture whole blood. Since the children in this sample were relatively young and because caregivers expressed hesitation about conducting venipuncture on their children, I did not collect plasma samples from participants. McDade & Shell-Duncan (2002) and Cook et al. (1998) report on the validation of using dried blood spots for analyzing sTfR for an in-house assay and a commercially available enzyme immunoassay kit from Ramco Laboratories, respectively. However, because the validated kit is no longer produced, I performed validation tests comparing plasma, venipuncture dried blood spots and fingerstick dried blood spots from nine American UNC affiliates using R&D Systems: Quantikine Human sTfR Immunoassay at the Human Biology Laboratory at the University of North Carolina, Chapel Hill. Due to the small sample size, I did not utilize the calculated ratio for sTfR in dried blood spots to sTfR in plasma in my studies. The protocol and results of my validation are reported in Appendix A.

Anthropometric Variables

Anthropometric measurements for all child participants are incorporated in analysis as indicators of nutritional status. These measurements were based on techniques described by Gibson (1990). At the initial and final interviews, I measured height to the nearest millimeter with a stadiometer while participants stood on a level platform (without footwear) and weight using a SECA electronic digital LED scale (without footwear and wearing light clothing). Waist circumference was measured with non-elastic tape midway between the lowest rib and the iliac crest. Tricep skinfold thickness (TSF) was measured to the nearest 0.5mm with precision Lange calipers.Measures of weight-for-age, height-for-age, tricep skinfold thickness, and body mass index z-scores were converted based on the WHO standard using the WHO 2007 STATA macro package (de Onis et al. 2007). High and low measures were dichotomized, children with greater than one standard deviation above zero were categorized as having high measures of body fat. Waist to height ratio (WHtR) was calculated by dividing the participant's waist circumference by height, if a score was at or above 0.5 the child was categorized as having a high ratio (Ashwell and Hsieh 2005).

Immune Function and Pathogenic Exposure

This study employed both subjective and objective measures of child morbidity as proxies for pathological exposure and immune function. Reported child morbidity indices are based on maternal reports of child illness within the two weeks prior to the initial (for all child participants) and final (for anemic participants) interviews. The presence or absence of "common cold" symptoms is based on the cumulative illness burden of runny nose and cough while the presence of respiratory infection symptoms are the cumulative burden of difficulty and/or rapid breathing.

This study also uses CRP as a direct measure of morbidity. CRP is an acute phase protein involved in the innate immune response (Ballou and Kushner 1992), thus the concentration of CRP increases in response to inflammation providing an indicator of morbidity (Rousham et al. 1998). Dried blood spots were analyzed from the initial interview for the total sample and from the final interview for children who received iron supplementation. All dried blood spot samples were placed in a freezer at -30°C at the health center in San Juan de Lurigancho until they were transported to the United States and placed in another -30°C freezer until analysis. Samples were exposed to above freezing temperatures for less than 24 hours, within the limits necessary to maintain sample integrity for CRP analysis (McDade et al. 2004). The dried blood spot samples were analyzed for CRP using an adapted enzyme-linked immunosorbent assay (ELISA) protocol for R&D Systems: Quantikine Human CRP Immunoassay at the Human Biology Laboratory at the University of North Carolina, Chapel Hill. CRP levels were dichotomized as elevated (<3 mg/dL) and not elevated (≥3 mg/dL) (Pearson et al. 2004) for both groups for chapter 4 and a 2.2 mg/L cut-off value to define children with low versus high concentrations of CRP was utilized for chapter 5 and 6 (Caminiti et al. 2016; Wander et al. 2009). I used different cut-off points for CRP in chapters 5 and 6 because analyses focused on anemic children as opposed to the entire sample.

Additionally, anemic children were invited to have parasite tests completed by the local health center, only 18 mothers opted to have their child complete parasitic testing. If the samples included evidence of parasitic infection, including *Enterobius vermicularis* (human pinworms) and *Giardia duodenalis* (giardia), two parasites that can contribute to anemia, then the child was labeled positive for parasites.

I collected observational data through checklist and free-form notes among children receiving treatment at each home visit to create a set of variables. These variables included playing with animals and eating dirt, both dichotomous variables (yes and no), and child bathroom practices (toilet, *bacin* (chamber pot), or a diaper for elimination). Age, sex, reported consumption of dirt as well as season of the initial interview were also included as these they both indicate differential contact with environmental and pre-school-related disease ecology and are associated with child response to supplementation. Two distinct seasons can be identified in Lima: summer, from December through April; and winter from May through November.

Gut Microbiota

If a child was anemic, mothers were asked to collect a small stool sample and store it in the provided containers before treatment began and after treatment ended. If the household had a working freezer the samples were stored there until the next morning when I retrieved the sample (typically less than 24 hours). If the household did not have a working freezer, mothers were asked to bring the sample to the community health center immediately upon collecting the sample. The stool samples were stored in a -25°C freezer at the health center until I transported them to the University of North Carolina – Chapel Hill at the completion of fieldwork.

The UNC School of Medicine Microbiome Core Laboratory analyzed samples by isolating DNA using Qiagen BioRobot Universal (Qiagen, Valencia, California) and with the Qiagen Blood and Tissue, and modified QIAmp DNA Stool protocols to ensure isolation of DNA from Gram-positive and Gram-negative bacteria (Thompson et al. 2015). This analysis included initial amplification of the V3-V4 region of the bacterial 16S gene on samples (Devine et al. 2013). The 16S rDNA amplicons were sequenced on a Roche GS FLX Titanium instrument (Microbiome Core Facility, Chapel Hill, North Carolina) and analysis of sequencing data was carried out using QIIME pipeline (Caporaso et al. 2010). For quality control, raw sequencing data were filtered and denoised (Reeder and Knight 2010). Sequences were grouped into Operational Taxonomic Units (OTUs) at a 97% level using Uclust (Edgar 2010). I used alpha and beta diversity measures calculated using QIIME to quantify microbiome community structure and we measured the observed species per sample phylogenetic diversity (PD) to provide a measure of taxa diversity. I focused analysis on taxa abundance at the phyla and order level for taxa having >1% abundance but mention taxa significant in bivariate models here because it is unknown whether these low bacterial abundances would produce a biological impact (McClorry et al. 2018).

Additional Child-Level Variables

At the second home interview, diet was investigated through food diversity measures. This measure is based on a six-month food diversity scale that included food items rich in heme and non-heme iron as well as vitamin C (aids in iron absorption) and calcium (an inhibitor of iron uptake). Due to the small sample size, only heme and leafy green variables were included in statistical analysis. The Iron Rich: Heme variable is a cumulative measure of red and organ meats as well a chicken blood. Due to non-normal distribution, the food frequency variables were dichotomized for all food variables. The heme variable was dichotomized by consumed or never having consumed foods rich in iron within the past six months. Consumption of leafy greens was dichotomized by eating these vegetables less than one time a week or more based on distribution of data.

At the final interview, adherence to treatment for anemia was assessed through a survey on the number of days the child was given treatment and through open-ended questions about the perceived effects of the ferrous-sulfate syrup and the ease or difficulty in giving treatment. Due to the non-normal distribution of days adhered, compliance was dichotomized based on the median number of days for the sample, children receiving treatment at least once a day for 22 days or more and children receiving treatment at least once a day for 21 days or fewer. Interview responses were coded for positive and negative associations of treatment for both perceived effects and giving treatment.

Maternal Variables

Maternal-level variables included measures of socio-economic status (maternal education, working outside of the home, and knowledge of anemia) and personal nutritional status (Hb concentration) and child nutritional status (perceived child size). Information on all variables, except for knowledge of anemia, was collected from all of the maternal-child dyad participants. All maternal variables are dichotomized by yes or no responses to survey questions except Hb concentration, maternal age, and perceived child size. Maternal Hb concentration, like child Hb, was measured using Hemocue Hb 201+ and was only conducted during the initial interview. Perceived child size includes the categories: smaller than average, average, and above average when compared to peers. Knowledge of anemia was assessed at the first home interview, and is therefore only available for anemic children. This dichotomous variable (yes or no) is based on whether the mother responded in affirmative or negative when asked if she could describe anemia and its effects on child growth.

Household and Environmental Variables

Variables for nutritional status and exposure to disease were also created at the household and environmental levels. Nutritional status measures included weekly food expenditure, food security status, and markers of socio-economic status (monthly income range, maternal grandmother's first language, living with maternal or paternal grandparents, and persons per bedroom). Exposure to pathogens at the household and environmental level was assessed using variables for pets, trash disposal, and season of interview. Categorical household-level variables for all participants include the maternal grandmother's first language as a proxy for ethnicity

(Spanish or Indigenous), living with maternal or paternal grandparents, and monthly income range (<750, 750-1500, >1500 soles).

Variables for just the anemic children included food insecurity, person per bedroom ratio, household elimination of trash, and the presence or absence of pets living in the household. Food insecurity is based on the Household Food Insecurity Access Scale (HFIAS). I dichotomized this measure into food secure if the respondents scored 15 and below. The person per bedroom variable is the ratio of number of people living in a household to the number of bedrooms present in the house. Household elimination of trash is a dichotomous variable (placed outside the house or taken by a garbage collector) that represents proximity to waste piles. Households that dispose of their trash in piles outside are closer to neighborhood garbage piles while those that hire a garbage collector tend to live farther from the public dumping grounds.

STATISTICAL ANALYSIS

Chapter 4 (Dissertation Article 1)

I used logistic regression to test the relationship between aspects of the child's microniche (child, maternal, household, and environmental predictor variables) and whether a child was anemic or not, and if anemic, whether the child responded to iron supplementation or did not respond to treatment. The general analytic strategy was to first examine the bivariate relationship between hypothesized predictor variables and anemia status/response to iron supplementation using logistic regression models. Following these analyses, I identified those variables that were significant at p-value .20, due to the small sample size, and used these variables in multivariate logistic modeling. Models were created at three levels: child, maternal, and household and environmental. These three models were used to test the hypothesized predictor variables that were significant at the bivariate level. Variables that did not remain significant at p-value .20 in each multivariate model were excluded in the final multivariate logistic model. The final model was built from all of the predictor variables that remained significant at a p-value of .20. Statistical analyses were conducted with STATA 13. In all logistic regression models, ages, sex, and season were included as covariates and robust standard errors accounting for clustering by maternal identification were used. This process adjusts for siblings included in the sample and provides a more conservative estimate of variance within the models.

Chapter 5 (Dissertation Article 2)

I first used bivariate logistic regression models to assess the relationships between response to iron supplementation, immune activation, and body size variables. To test the hypothesis that immune activation is associated with reduced ability to respond to iron supplementation, I investigated the association with elevated CRP with changes in hemoglobin concentration over one month of iron supplementation in multivariate logistic regression models including age, sex, and season of data collection. I evaluated a wider range of individual, maternal, household, and environmental variables in previous analyses (Chapter 4), but none approached significance as predictors of response to iron supplementation and are therefore not included as covariates here.

To test the hypothesis that body fat moderates the impact of immune activation on response to iron supplementation, I included several body fat variables in the additional multivariate logistic models. Moderation was tested through the inclusion of interaction terms representing the interaction between immune activation and body fat measures (high WHtR, TSF z-score, and BMI z-score) at the final interview. Statistical analyses were conducted with STATA 13. Like in Chapter 4, all logistic regression models, ages, sex, and season were included as covariates and robust standard errors accounting for clustering by maternal identification were used. This process adjusts for siblings included in the sample and provides a more conservative estimate of variance within the models.

Chapter 6 (Dissertation Article 3)

I tested for differences in alpha and beta diversity between pre and post samples as well as within both timing groups by response to supplementation using QIIME. I also tested for variation in taxa abundance using regression models for each of the aforementioned pairings. Initially these models included just the taxa in question and timing or response variables. For taxa with and abundance greater than 1% and showing evidence of difference (p-value<0.25), I added pathogenic exposure variables to the model to test whether those differences persisted. Additional descriptive statistics and bivariate tests (chi-square tests for categorical variables and T tests for continuous variables) were employed to further explore the relationship between taxa and other variables of interest. All models were adjusted for siblings in the samples by clustering by maternal identification number. All analyses were completed using STATA 13.

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CHAPTER 4. PREDICTORS OF ANEMIA AND RESPONSE TO IRON SUPPLEMENTATION

Child, caretaker, and community: Testing predictors of anemia and response to iron supplementation in Peruvian pre-school-aged children

INTRODUCTION

Despite reductions in rates of poverty and child hunger, anemia remains a widespread public health problem. Anemia is caused by low levels of hemoglobin (Hb), a protein vital to the transportation to and storage of oxygen in organs and tissue, and iron deficiency is considered the leading cause of anemia globally (Stoltzfus et al. 2004). Established risk factors for iron deficiency anemia include physiological (e.g. age and sex), nutritional (e.g. low iron consumption), and pathological (e.g. blood loss, inflammation, and malabsorption) conditions (Lopez et al. 2016). Anemia affects roughly 25% of the world's population and an estimated 293 million children of preschool age are anemic (WHO 2009). Anemia has crucial implications for long term health. Childhood anemia can cause delayed and decreased cognitive and physical development and function (Stoltzfus et al. 2004a). The consequences of these symptoms are associated with loss of productivity including reduced work capacity, cognitive impairment, and increased susceptibility to infection (Balarajan et al. 2011).

Due to concerns about anemia's negative impact on child development, global health institutions recommend iron supplementation and fortification of all children in populations with a high prevalence of anemia (World Health Organization [WHO] 2002; Stolzfus and Dreyfuss 1998). Supplementation and fortification have been proven to be effective public health interventions to reduce anemia rates (Thompson et al. 2013; Zimmermann and Hurrell 2007; Baltussen et al. 2004). A comprehensive review of the efficacy of iron supplementation concluded that the majority of randomized-control-trials investigating the effectiveness of iron supplementation in children report significant increases in Hb concentration and other iron status indicators as well as reduced anemia prevalence (Iannotti et al. 2006). When iron supplementation has not been effective in reducing anemia individually, experts suggest investigating poor compliance (Galloway and McGuire 1994) and malabsorption (Lopez et al. 2016).

Peru suffers from high anemia rates, making it similar to most sub-Saharan African countries, where anemia rates tend to be highest (Alcázar 2013). The WHO (2009) has categorized Peru as having "severe" anemia prevalence and estimates that about half of all pre-school age children, pregnant women, and non-pregnant women of reproductive age suffer from anemia. The prevalence of anemia in children under five is higher than the prevalence of malnutrition and has remained constant despite decreases in rates of stunting and poverty (Marini et al. 2017). The high rates of anemia in Peru have been subject to considerable governmental and non-governmental attention and several initiatives have aimed to reduce levels of anemia, specifically among children five years and younger, including the most recent campaign *Plan Nacional para la Reducción de la Anemia* 2017-2021 (National Plan to Reduce Anemia). Despite these repeated interventions for reducing anemia and malnutrition more broadly, anemia continues to represent a distinct challenge, even for those living near large cities who have greater access to urban infrastructure.

San Juan de Lurigancho is a peri-urban district in the north-east quadrant of Lima, the capital and largest city in Peru, has been the site of numerous anemia interventions to date. In 2013, 35.7% of children under five living in this district were diagnosed with anemia; in 2014, despite the efforts of the Ministry of Health and community-based organizations, the percentage had *increased* to 41.9% (Ministerio de Salud 2015). San Juan de Lurigancho provides an ideal context to investigate anemia and iron supplementation due to the high rates of anemia, the lack of success among programs that seek to combat this health concern, and the expressed anxiety about anemia from community members.

This article reports a study of the biocultural aspects of anemia in a sample of pre-school-aged children living in San Juan de Lurigancho. The concept of the developmental microniche (Super and Harkness 2002; Worthman 1994) is a useful model for exploring the relationship between socioecological context and health. The niche is defined as the variable individual context of each child, and includes the social as well as physical settings in which each child develops (Harkness and Super 1986; Worthman 2010). The niche provides a level of analysis and explanation between the more proximate (physiological) and distal (social) aspects of biology, and a framework to organize and explore relationships between children's biology and their socio-ecological context (e.g., Brewis 2003). The developmental microniche, therefore, serves as a useful guide for methods and analyses to explore how individual anemia status and efficacy of iron supplementation can be linked to national campaigns.

Here we aim to contribute to the growing knowledge of anemia status and iron supplementation through an investigation of 102 pre-school-aged children living in a Peruvian peri-urban community. Our first goal is to explore the overall prevalence of anemia and to identify aspects of the developmental microniche that are associated with anemia status. Our second, related goal, is to consider how national level campaigns to reduce anemia are mediated through socio-ecological factors at a more proximate (individual) level by investigating the efficacy of iron supplementation within this particular context. We hypothesized that anemia status and response to supplementation would be related to similar child, maternal, household, and environmental variables that likely indicate increased iron requirements (e.g. age and sex), diet and nutritional status (e.g. weight, height, food frequencies, socio-economic status), and exposure to disease (e.g. reported morbidity symptoms, C-reactive protein, parasitic infection, and season of enrollment).

MATERIALS AND METHODS

Study setting and sample

Peri-urban communities around Lima, like the ones found in San Juan de Lurigancho, are the result of policies that favored highland landlords over rural peasants prior to Peru's agricultural reforms and the promise of higher wages available in the expanding capital, the growth and number of these unplanned communities increased dramatically in the 1980s and early 1990s when violence in the highlands perpetrated by *Sendero Luminoso* (Shining Path) and the state's brutal counter-insurgency forces drove another influx of migrants to Lima (Theidon 2010, Seligmann 2004). During this period, over 600,000 peasants emigrated from the highlands (Theidon 2010). While many of the residents of these communities escaped direct violence, internal migrants such as those who settled in San Juan de Lurigancho nonetheless experience severe stigma and remain in communities characterized by poor quality or informal housing, unhealthy living conditions, poverty, and marginalization from the formal health sector resulting in severe health inequalities (Hetzinger 2014).

The study sample is represented by 102 children (50 girls and 52 boys), ages 2-5 years, living in a neighborhood of San Juan de Lurigancho and represents a relatively disadvantaged area of the Lima district. While many parents described themselves and their children as *mestizos* (having Spanish and indigenous descent) and *Limeños* (native of Lima, Peru), many occupants of wealthier Lima districts (e.g. Miraflores and La Molina) contradicted this description, claiming the families living in San Juan de Lurigancho were indigenous, coming from the Andes or the

Amazon regions, and often added stories of alcoholism, theft, domestic violence, and child neglect that plague the San Juan de Lurigancho district. This stigma associated with San Juan de Lurigancho is recognized by its inhabitants and many caregivers expressed a desire for their children to become professionals to rise above this reputation. One example of the desire for upward mobility is the language shift occurring in peri-urban communities around Lima. While many grandmothers spoke Quechua, an indigenous Andean language, exclusively, all children in this sample spoke only Spanish. This generational loss of Quechua may be due to the stigma associated with the indigenous language and the belief that bilingualism would prevent sought after social mobility (Hornberger and Coronel-Molina 2004).

Study Design and Data Collection

Between November 2017 and July 2018, the first author collected qualitative and quantitative data from caregivers and anemic preschool-age children and their caregivers living in three neighborhoods within the San Juan de Lurigancho district of Lima, Peru. Research in this community was conducted in collaboration with researchers at the *Instituto de Investigación Nutricional* (IIN), a private, non-profit institution dedicated to interdisciplinary research on health and nutrition in Peru. This study received both University of North Carolina at Chapel Hill IRB and IIN Ethics Board approval for this project.

The study field coordinator and first author recruited 102 caregivers (typically mothers) and their children (aged 2-5 years) through door-to-door and "snowball" sampling (Figure 4.1). All maternal-child dyads participated in an initial interview at the center that included Hb assessment. The initial interview took place in a small private room at the local health center that included an extensive demographic survey, morbidity symptom report, and anthropometric measurements. At the conclusion of the interview, the child's capillary Hb was measured to test for anemia via a minimally invasive finger prick and a portable photometer Hemocue machine

(Hemocue Hb 201+, HemoCue America, California). The child's finger was cleaned with alcohol, and a sterile disposable microlancet was used to deliver a controlled puncture. Whole blood was placed directly onto the Hemocue cuvette and inserted into the Hemocue machine. The Hb measurement was conducted within 30 seconds of the finger prick and was used to determine if the child was or was not anemic using the World Health Organization's definitions of iron status (WHO 2009). At least one drop of blood was collected from participants after the finger prick for Hb measurement on standardized filter paper (Whatman #903. Middlesex, UK) for analysis of C-reactive protein (CRP), a direct measure of immune activation.

Half of the participants were not anemic (n=51) and were excluded from further interviews. If the child was anemic, the participant visited the health center pediatrician and was prescribed ferrous sulfate in syrup, a treatment with a high concentration of iron. Treatment included consuming a tablespoon of ferrous-sulfate syrup two times a day, once in the morning and once in the afternoon at least 30 minutes before a meal for 28 days. This treatment followed the anemia intervention guidelines provided by the Peruvian Ministry of Health (Ministerio de Salud 2015). The maternal-child dyad participated in three additional interviews over the course of one month, the time period during which the child should respond to the treatment.

Two of the additional interviews took place in participant's homes in a room of the caregivers' choosing (most often the kitchen or living room). These interviews included reports of child, maternal, and household demographic characteristics and estimates of child health status, mother's perceptions of child size, and dietary patterns. Considerable time was spent in ethnographic observation during the home visits and would typically include playing with the child, eating meals with the family, and walking the child to school.

The final interview took place in a private room at the local health center after four weeks of treatment. This interview included a morbidity symptom report, open-ended questions about compliance to the treatment protocol, and a final Hb test using Hemocue Hb 201+ to assess the child's response to treatment. Another spot of blood was collected from participants after the finger prick to assess CRP levels after one month of treatment.

All dried blood spot samples were placed in a freezer at -30°C at the health center in San Juan de Lurigancho until they were transported to the United States and placed in another -30°C freezer until analysis. Samples were exposed to above freezing temperatures for less than 24 hours, within the limits necessary to maintain sample integrity for CRP analysis (McDade et al. 2004). The dried blood spot samples were analyzed for CRP using an adapted enzyme-linked immunosorbent assay (ELISA) protocol for R&D Systems: Quantikine Human CRP Immunoassay at the Human Biology Laboratory at the University of North Carolina, Chapel Hill.

Child Variables

In the following analyses, anemia is used as a dichotomous variable (not-anemic ≥11.0g/dL and anemic <11.0g/dL). These categories are based off of WHO recommendations (2011), which have not changed since 1968 but continue to be used in a number of studies and publications. Response to iron supplementation is also used as dichotomous variable (responder and non-responder). Children who became not anemic (≥11.0 g/dL) after 1 month of treatment were labeled as responders while children whose Hb level remained below 11.0 g/dL at the final test were categorized as non-responders.

Anthropometric measurements for all child participants are incorporated in analysis as indicators of nutritional status. These measurements were based on techniques described by Gibson (1990). At the initial and final interviews, the first author measured height to the nearest millimeter with a stadiometer while participants stood on a level platform (without footwear) and weight using a SECA electronic digital LED scale (without footwear and wearing light clothing). Measures of weight-for-age and height-for-age to z-scores were converted based on the WHO standard using the WHO 2007 STATA macro package (de Onis et al. 2007). Adiposity is based on each participant's waist to height ratio; if a score was at or above 0.5 the child was categorized as having high central adiposity (Ashwell and Hsieh 2005).

Due to the study design, more information was collected and incorporated into analysis for the anemic children. At the second home interview, diet was investigated through food diversity measures. This measure is based on a six-month food diversity scale created by the IIN that included food items rich in heme and non-heme iron as well as vitamin C (aids in iron absorption) and calcium (an inhibitor of iron uptake) to assess diversity of diet. Due limited variation in many of the variables, only heme and leafy green variables were included in statistical analysis. The Iron Rich: Heme variable is a cumulative measure of red and organ meats as well a chicken blood. Due to non-normal distribution, the food frequency variables were dichotomized for all food variables. The heme variable was dichotomized into never and consumed foods rich in iron within the past six months. Consumption of leafy greens was dichotomized by eating these vegetables less than one time a week or more based on the distribution of the data.

At the final interview, adherence to treatment for anemia was assessed through a survey on the number of days the child was given treatment and through open-ended questions about the perceived effects of the ferrous-sulfate syrup and the ease or difficulty in giving treatment. Due to the non-normal distribution of days adhered, compliance was dichotomized based on the median number of days for the sample, children receiving treatment at least once a day for 22 days or more and children receiving treatment at least once a day for 21 days or fewer. Interview responses were coded for positive and negative associations of treatment for both perceived effects and giving treatment.

This study employed both subjective and objective measures of child morbidity as proxies for pathological exposure. Reported child morbidity indices are based on maternal reports of child illness within the two weeks prior to the initial (for all child participants) and final (for anemic participants) interviews. The presence of “common cold” symptoms is based on the cumulative illness burden of runny nose and cough while the presence of respiratory infection symptoms are the cumulative burden of difficulty and/or rapid breathing. This study also uses CRP as a direct measure of morbidity. CRP is an acute phase protein involved in the innate immune response (Ballou and Kushner 1992), thus the concentration of CRP increases in response to inflammation providing an indicator of morbidity (Rousham et al. 1998). Dried blood spots were analyzed from the initial interview for the total sample and from the final interview for children who received iron supplementation. CRP levels were dichotomized as elevated (<3 mg/dL) and not elevated (≥3 mg/dL) (Pearson et al. 2004) for both groups. Additionally, anemic children were invited to have parasite tests completed by the local health center, only 18 mothers opted to have their child tested for parasitic load. If the samples included evidence of parasitic infection, including *Enterobius vermicularis* (human pinworms) and *Giardia duodenalis* (giardia), two parasites that can contribute to anemia, then the child was labeled positive for parasites. Observational data from children receiving treatment included playing with animals and eating dirt, both dichotomous variables (yes and no), and child bathroom (the child uses a toilet, *bacin* (chamber pot), or a diaper for elimination).

Maternal Variables

Maternal-level variables included measures of socio-economic status (maternal education, working outside of the home, and knowledge of anemia) and personal nutritional status (Hb concentration) and child nutritional status (perceived child size). Information on all variables, except for knowledge of anemia, was collected from all of the maternal-child dyad participants. All maternal variables are dichotomized by yes or no responses to survey questions except Hb concentration, maternal age, and perceived child size. Maternal Hb concentration, like child Hb, was measured using Hemocue Hb 201+ and was only conducted during the initial interview. Perceived child size includes the categories: smaller than average, average, and above average when compared to peers. Knowledge of anemia was assessed at the first home interview, and is therefore only available for anemic children. This dichotomous variable (yes or no) is based on whether the mother responded in affirmative or negative when asked if she could describe anemia and its effects on child growth.

Household and Environmental Variables

Variables for nutritional status and exposure to disease were also created at the household and environmental levels. Nutritional status measures at the household level included weekly food expenditure, food security status, and markers of socio-economic status (monthly income range, maternal grandmother's first language, living with maternal or paternal grandparents, and persons per bedroom). Exposure to pathogens at the household and environmental level was assessed using variables for pets, trash disposal, and season of interview. Categorical household-level variables for all participants include the maternal grandmother's first language as a proxy for ethnicity (Spanish or Indigenous), living with maternal or paternal grandparents, and monthly income range (<750, 750-1500, >1500 soles). Season is a dichotomous variable based on the month in which the initial interview took place, summer (December-April) and winter (May-

November). Variables for just the anemic children included food insecurity, person per bedroom ratio, household elimination of trash, and the presence or absence of pets living in the household. Food insecurity is based on the Household Food Insecurity Access Scale (HFIAS). We dichotomized this measure into food secure if the respondents scored 15 and below. The person per bedroom variable is the ratio of number of people living in a household to the number of bedrooms present in the house. Household elimination of trash is a dichotomous variable (placed outside the house or taken by a garbage collector) that represents proximity to waste piles. Households that dispose of their trash in piles outside are closer to neighborhood garbage piles while those that hire a garbage collector live farther from the public dumping grounds.

Statistical Methods

We used logistic regression to test the relationship between aspects of the child's microniche (child, maternal, household, and environmental predictor variables) and whether a child was anemic or not, and if anemic, whether the child responded to iron supplementation or did not respond to treatment. The general analytic strategy was to first explore the bivariate relationship between hypothesized predictor variables and anemia status/response to iron supplementation using logistic regression models. Following these analyses, we identified those variables that were significant at p-value .20, due to the small sample size, and used these variables in multivariate logistic modeling. Models were created at three levels: child, maternal, and household and environmental. These three models were used to test the hypothesized predictor variables that were significant at the bivariate level. Variables that did not remain significant at p-value .20 in each multivariate model were excluded in the final multivariate logistic model. The final model was built from all of the predictor variables that remained significant at a p-value of .20. Statistical analyses were conducted with STATA 13. In all logistic regression models, ages, sex, and season were included as covariates and robust standard errors accounting for clustering by maternal identification were used. This process adjusts for siblings included in the sample and provides a more conservative estimate of variance within the models.

RESULTS

Demographic Characteristics – Total Sample

In our initial sample of 102 pre-school aged children, half were anemic. Of the anemic children, 63% suffered from mild anemia (Hb: 10.0-10.9 g/dL), and 37% experienced moderate anemia (Hb: 7.0-9.9 g/dL). Generally, children were above average for HAZ and despite being slightly below average for WAZ, the majority of participants had a high adiposity level (Table 4.1). Cold symptoms (runny nose and cough) were the most frequently reported morbidity symptom but only 20% of children had an elevated CRP at the initial interview.

The mean age of mothers in the sample was just over 30 years old and they were, on average, not anemic (Table 4.2). The majority of mothers had completed their high school education. While less than half of the mothers were working outside the home, their modal occupations were vendors selling food or clothing, cooks in restaurants or homes, and maids, many mothers discussed how their occupational status would change throughout the year. Mothers would often work before school began to make extra money for their child’s uniform, supplies, and fees. When asked about their child’s size in relation to their peers, almost half of all mothers reported perceiving their child as smaller than average.

Homes were typically multi-generational (60% of children in this sample shared homes with their maternal grandparents) with families occupying different floors and sharing the kitchen area (Table 4.3). The majority of maternal grandmothers spoke Spanish as a first language, indicating *mestizo* identity. Just under half of the households in this sample earned an average of 750-1500 soles per month (about $225-449 USD) and the average household spent 186.67 soles (about $56 USD) on food each week. Households had roughly 2-3 children under the age of five years and just over half of the sample participated in this study during the summer season.

Predictors of Child Anemia Status

At the bivariate level, sex was not significantly associated with anemia status (p-value: 0.70) but older children were less likely to be anemic than younger children (OR:0.96, CI:0.93-0.99, p-value: 0.01). While HAZ and adiposity were not associated with anemia status (p-value >0.26), WAZ was (OR: 0.63, CI:0.42-0.94, p-value:0.02). A variety of patterns were observed with variables representing child morbidity. If a mother reported a child as having diarrheal symptoms two weeks prior to the initial interview, the odds of the child having anemia increased (OR: 4.92, CI:1.29-18.77, p-value:0.02). On the other hand, if the child had cold symptoms the likelihood of the child being anemic decreased (OR: 0.61, CI:0.16-1.06, p-value:0.07). Loss of appetite, respiratory infection symptoms, and elevated CRP at the initial interview were not significantly associated with anemia status (p-value >0.42). When age, WAZ, diarrheal disease, and cold symptoms were placed in the multivariate child model, all of the aforementioned patterns persisted (Table 4.4).

The only maternal variables approaching significance at the bivariate level were Hb level (OR: 0.72, 0.51-1.02, p-value:0.07) and education (OR: 2.06, CI:0.89-4.80, p-value:0.09), but only Hb level remained significant in the maternal-level multivariate model (Table 4.4). Mothers with higher Hb concentrations were more likely to have a child that is not anemic than mothers with lower Hb scores. While maternal perceived child size in relation to other children did not predict child anemia status or response to iron supplementation in bivariate testing (OR: 0.97, CI: 0.71-1.31, p-value:0.83), mothers associated size and potential anemia status in interviews. One mother, after learning that her four-year-old daughter had a Hb concentration of 9.4 declared

“¡*Pero ella es gordita! ¿Como puede tener anemia*?” (But she is chubby! How can she have anemia?).

Household variables approaching significance at the bivariate level included living with paternal grandparents (OR:0.41, CI: 0.16-1.04, p-value: 0.06) and the number of children under 5 years of age living in the home (OR:1.25, CI: 0.90-1.74, p-value: 0.18). Season was also associated with anemia status at the bivariate level (OR: 2.46, CI: 1.10-5.53, p-value: 0.03). While living with paternal grandparents and season remained significant in the multivariate household and environmental model, the number of children below 5 years of age did not (Table 4.4).

All variables that remained significant at p-value 0.20 in their respective multivariate models were included in the final model. While their effect sizes reduced slightly from previous models, WAZ, living with paternal grandparents, and season remained significant predictors of anemia status (Table 4). As child WAZ increases by one standard deviation, the odds of having anemia was reduced by 48%. Participants living with their paternal grandparents have a reduced likelihood of being anemic compared to those living with their maternal grandparents. Children who participated in the study during the summer are more likely to be anemic than children enrolled during the winter.

Developmental Microniche of Child Anemia

Of the 51 children who were anemic at the initial interview, one participant was lost to follow-up because the father was promoted at work and the family moved out of San Juan de Lurigancho. When the 50 remaining children were tested at the final interview, 25 remained anemic. The average Hb concentration at the end of treatment was 10.87 (Table 4.1). Many children were still using diapers and 32% of mothers reported observing their child consume dirt in the past week. On average, households had roughly 3 people per bedroom and 66% of households had pets. Many of the houses were food secure and placed their trash outside for disposal. The majority of children exhibited cold symptoms in the two weeks prior to the final interview and 36% of mothers reported loss of appetite in their children. At the final interview, 28% of participants had an elevated CRP. Overall this sample had low levels of parasitic load. Three of the children in this sample tested positive for human pinworms and 13 tested positive for giardia.

Food consumption patterns reflect low dietary iron intake. There is a low frequency of the consumption of red and organ meat, foods with a high availability of iron. While almost all participants eat iron rich legumes like lentils and beans often (averaging twice a week), only 56% of children consumed leafy greens (another nonheme iron source) more than one time a week (Table 4.1).

Compliance to treatment was high, 52% of mothers reported adhering to treatment for 22 days or more. Adherence ranged from only receiving the medicinal syrup 2 days during the four-week treatment period to 28 days, the maximum number of days possible. Many mothers in the sample explained the lack of adherence to treatment to being preoccupied with other things and that their child did not like the taste of the syrup. A mother of a four-year-old boy described her mornings as "*ocupado*" (busy) "*porque tengo cuatro hijos que necesitan ir a la escuela y necesito ir a trabajar*" (because I have four children that need to go to school and I need to go to work). She also sighed, and declared "*si tuviera un hijo nunca me olvidaría de darle*" (If I had [only] one child, I would never forget to give it). This comment illustrates not only stress of mothers' daily responsibilities, something parents experience in nearly every human society, but also suggests that studies of iron supplementation need to consider the number of children at home—not just children with anemia.

Child reactions to iron sulfate ranged from liking the syrup and asking for more to running away or fighting with the caregiver who was trying to get the child to take their medicine. Several children described the taste of the syrup as "*feo*" (ugly) and did not want to eat it. In one case, a child participant arrived at an interview with a large bruise on their forehead. When asked what happened the mother described her daughter running away from her and then falling down the stairs when she tried to give her daughter the medicine one morning.

Some caretakers mentioned needing to use "*fuerza*" (force) to get their child to take the spoonful of syrup two times each day, and one mother admitted to becoming so frustrated with her four-year old son's resistance to opening his mouth that she threatened to make him "*como Túpac Amaru*." The mother was referring to Túpac Amaru II, a leader of an Andean uprising in the late 18th century. His torture and death included being drawn, quartered, and then beheaded in Cuzco's main plaza (Chamber and Chasteen 2010). While this may seem like a shockingly violent threat for a mother to make to her son, the child did not know the story of Túpac Amaru and continued to refuse to take the iron-fortified syrup.

Despite the difficulties of providing treatment, 67% of caregivers said that they believed the ferrous sulfate was successful in treating anemia in their children, and when asked for more clarification mothers often responded that the syrup was functioning because their child's appetite had increased. While the most often cited side effects from iron supplementation (constipation, stomach cramps, and nausea) are often associated with a decrease in appetite, iron supplementation has been shown to influence maternal perception of increased child appetite (Stolzfus et al. 2004).

Awareness of nutritional issues among mothers in this sample is high, 92% mothers reported knowing about the cause and/or health effects of anemia, this may be due in part to the successful efforts of the Peruvian state to decrease rates of stunting and malnutrition. These included communications strategies developed and implemented by NGOs that promoted understanding and awareness of the causes of and preventative measures for malnutrition (Marini et al. 2017). The relationship between diet and health is well known within this sample, and caregivers repeatedly linked inadequate (the most common examples included: not having enough food and poor appetite) and unhealthy food (such as fried foods and sweets) to disease and illness.

Predictors of Response to Iron Supplementation

While there are number of similar predictors between anemia status and response to iron supplementation, several differences were also noted. In bivariate modeling, Hb concentration at the initial interview was not a significant predictor of whether or not the participant responded to treatment (OR: 1.22, CI: 0.56-2.56, p-value: 0.62), responders included children with both mild and moderate anemia (Figure 4.2). Higher WAZ (OR: 1.91, CI: 1.08-3.38, p-value: 0.03), HAZ (OR: 1.74, CI:1.01-3.00, p-value: 0.05), and adiposity (OR:4.47, CI:0.78-25.76, p-value 0.09) were all associated with greater odds of responding to iron supplementation. While CRP at the initial interview does not have a significant effect on whether or not children responded to treatment (OR:0.35, CI:0.08-1.59, p-value:0.17), children with elevated CRP levels at the final interview were less likely to respond to treatment (OR:0.29, CI:0.08-1.07, p-value: 0.06). Parasitic infection did not predict response to treatment (OR:0.65, CI: 0.16-2.56, p-value 0.54). Children who eat leafy greens (e.g. spinach) more than one time a week have a greater likelihood of responding to treatment than those who do not (OR:2.70, CI:0.84-8.74, p-value:0.10). Consuming heme sources of iron at any point in the past six months (OR:5.41, CI:1.02-27.71, p-value:0.05) and observed geophagy (OR:3.14, CI:0.85-11.63, p-value:0.09) increased a child's odds of responding to treatment dramatically, these predictors were excluded from subsequent models due to small sample size. Within the child-level model, only two of the patterns observed in the bivariate models persist, WAZ and final CRP (Table 4.5).

Only one maternal variable was a significant predictor of child response to iron supplementation at the bivariate level. If a mother completed her high school education, her child had a lower likelihood of responding to iron supplementation than if a mother had not completed secondary school (OR:0.31, CI:0.08-1.16, p-value: 0.08). In addition to being a significant predictor of child anemia status, season was also a significant predictor for response to iron supplementation at the bivariate level (OR:0.13, CI:0.03-0.53, p-value: 0.01), however, living with paternal grandparents and the number of children were not (p-value >0.58). The ratio of persons to bedroom was associated with response to iron supplementation, a larger ratio was predictive of not responding to treatment (OR:0.61, CI:0.37-0.99, p-value:0.05). Both the person-to-bedroom ratio and season remained significant in the household and environmental multivariate model (Table 4.5).

All significant predictors or variables approaching significance from each of the three levels were placed in a final multivariate model. WAZ, CRP at the final interview, and season all remained significant predictors of responding to iron supplementation (Table 4.5). As WAZ increased, the probability of responding to treatment also increased. Participants with elevated CRP at the final interview were less likely to respond to supplementation than those who did not have elevated CRP. If a child is treated for anemia during the summer, the probability of the child responding to treatment decreases by 92%.

DISCUSSION

While anemia is a global health problem, effective strategies for anemia reduction require an understanding of context-specific causes and interventions that address predictors effectively. This paper aims to contribute to our growing knowledge about anemia as well as iron supplementation among preschool aged children in a community within San Juan de Lurigancho. Our first goal was to explore the overall prevalence of anemia and to identify aspects of the developmental microniche that are associated with anemia status. Half of all children in this sample were anemic, making the prevalence of anemia in this community higher than the national, Lima region, and Lima district rates (43.6%, 33.2%, and 26.4%, respectively) (ENDES 2017). The only area within Peru that reports an anemia prevalence higher than that in this study is Puno, a city located near Lake Titicaca at an altitude of 3,830 meters (ENDES 2017). While a number of variables were predictive of anemia status at the bivariate level, only three remained significant in the final model. We found that heavier participants, children living with their paternal grandparents, and those who enrolled in the study during the winter were less likely to be anemic than their counterparts.

We also wanted to consider how national level campaigns to reduce anemia are mediated through socio-ecological factors at a more individual level by investigating the efficacy of iron supplementation within this particular context. Of the 50 children diagnosed with anemia at the beginning of our study, only 25 responded to iron supplementation treatment. This result is counter to the findings described in a review article that included a meta-analysis of 21 data sets from randomized-control-trials (RCTs) exploring iron supplementation in children 0 to 12 years-of-age (Ramakrishnan et al. 2004). Ramakrishnan et al. (2004) found a significant difference in the mean change in Hb concentrations between treatment and control groups (OR:1.49, CI:0.46-2.51, p-value<0.05). A more recent comprehensive review of the efficacy of iron supplementation concluded that the majority of RCTs investigating the effectiveness of iron treatment in children report significant increases in Hb concentration and other iron status indicators as well as reduced anemia prevalence (Iannotti et al. 2006). The differences between our current results and the findings presented in the aforementioned review articles may be due to variation in the developmental microniche of each child, specifically nutritional status and exposure to disease.

Like anemia status, there were a number of socio-ecological factors that predicted response to iron supplementation at the bivariate level, but only three remained significant in the final model. A lower WAZ, an elevated CRP at the final interview, and being enrolled in the study during the summer season were associated with a reduced likelihood of a child responding to treatment.

We hypothesized that anemia status and response to supplementation would be related to similar child, maternal, household, and environmental variables that likely indicate increased iron requirements, poorer diet and nutritional status, and increased exposure to disease. WAZ and season were the only two variables the remained significant predictors for both anemia status and response to iron supplementation. Heavier children were more likely to have positive health outcomes, they were less likely to be anemic and, if anemic, more likely to respond to iron supplementation.

Results from research investigating the relationship between weight and anemia status are mixed. Some studies report that higher body mass index (BMI) results in an increased risk for iron deficiency and anemia among children and adolescents in both high income and transitioning settings (Aberli et al. 2011; Eftekhari et al. 2009; Zimmermann et al. 2008; Nead et al. 2004). While other studies have observed lower rates of anemia in women and children experiencing overnutrition (Kroker-Lobos et al. 2011; Eckhardt et al. 2008). Zimmerman et al. (2008) investigated the relationship between weight and anemia status as well as iron fortification. They report that increased adiposity in women and children results in lower anemia prevalence as well as a reduced response to iron fortification. In a study examining the efficacy of iron supplementation, Baumgartner et al. (2013) report that South African children with high BMI-for-age-z-scores have a greater risk for remaining iron-deficient after iron supplementation for 8.5 months when compared to children with low BMI-for-age-z-scores. Our results add evidence for the positive association between weight and anemia status but are contradictory to the findings on weight and iron supplementation presented by Zimmerman et al. (2008) and Baumgartner et al. (2013). This contradiction may be due to differences in variables (WAZ vs BMI) and/or the complex set of environmental and individual variables that include differences in disease exposure and immune activation caused by specific economic and cultural contexts.

Our statistical results are also reflected in the qualitative data, with mothers associating smaller body size with poor health and an increase in child appetite as a signal that they were recovering from anemia. This demonstrates that caregiver impressions of child body size play an important role in whether caregivers believed their child was healthy or not. These results are comparable to conclusions from previous research on infants feeding practices. A study conducted by Heinig et al. (2006) report ethnographic evidence from sixty-five Women, Infants and Children (WIC) eligible participants that demonstrate a positive association between higher infant weight and health in infants. Thompson and Bentley (2013) document that the cultural belief that “greedy” infants are healthier influence maternal feeding practices among first-time, low- income African-American mothers in central North Carolina participating in the Infant Care Study. The strong association between size and wellbeing in our sample may be due to cultural beliefs related to the aforementioned successful campaigns to reduce stunting in Peru. Only 19% of children in this sample were overweight, however, 49% of the sample were stunted. Stunting has been shown to be associated with increased risk of obesity in childhood (Wells et al. 2020;

Popkin et al. 1996). Therefore, the positive association between larger body size and health may lead to difficulty in recognizing nutritional deficiencies as the child ages.

The second variable that remained a significant predictor for both child anemia status and response to iron supplementation in our study was season. The summer season (December-April) was associated with the more negative health outcomes, children enrolled in the study during the summer season were more likely to be anemic and less likely to respond to iron supplementation. Seasonal variation in anemia rates has been documented by several authors (Senn et al. 2010; Rogerson et al. 2000), however these studies link Hb fluctuations to increased rates of malaria during the rainy season in Papua New Guinea and Malawi, respectively. However, not only is there a lack of precipitation year-round, malarial parasites are not found in the urban areas of Lima and therefore not the cause of the seasonal patterns documented in this study. Seasonal distribution of rotavirus, a virus linked to severe gastroenteritis and a potential cause of anemia, has been documented in District of Independencia, but the prevalence of rotavirus peaked during the winter months in this peri-urban community within Lima and rates of this virus have decreased since the introduction of the rotavirus vaccine (Chang et al. 2015). While our current results demonstrate a lack of association between diarrheal disease and negative health outcomes, additional investigation is needed to determine if the driving force of the seasonal variation observed in this study is due to disease ecology.

Fluctuations in diet may also contribute to the differences observed in anemia status and response to iron supplementation between the winter and summer months. While seasonal variation in diet has been documented in highland Peru, children experienced little seasonal change in in energy intake (Leonard and Thomas 1989). However, a study investigating child (0-35 months) nutritional status in Pampas de San Juan de Miraflores from 1987-1993, report seasonal variation, the mean weight-for-height was an estimated 0.38 z-score higher in the winter than in the summer (Marin et al 1996). The negative outcomes associated with the summer season (anemia and non-response to supplementation) in this study may be linked to significant variation in nutritional status.

While markets provided a wide-range of foods all year round, children were enrolled in school during the winter months. Based on the preliminary investigation of 24-hour dietary recalls, diet patterns in the summer months were unstructured compared to the winter season. Meal times varied day to day and snacks were more frequent in the summer. In the winter, all children in the sample were enrolled in pre-school, and the days became more structured. Breakfast and lunch were dictated by the start and end of the school day. Seasonal variation in anemia and response to treatment in our sample may be due to differences in the composition and structure of child diet related to enrollment in school. Eating sporadically and less frequently may cause depletion of vital nutrients and minerals, resulting in higher rates of anemia during the summer.

While the initial level of CRP did not remain a significant predictor of response to iron supplementation, having an elevated CRP at the final visit remained significant with WAZ, maternal education, persons-per-bedroom ratio, and season covariates. These results demonstrate an interesting relationship between levels of inflammation and anemia status. While earlier studies report opposing results on the association between morbidity and anemia (Wander et al. 2009; Hadley and DeCaro 2015), a more recent study investigating the association between infection and anemia status within the San Juan de Lurigancho community found that while morbidity was not predictive of anemia status over a six-month interval period, it was shown to be associated with current morbidity symptoms (Dorsey et al. 2018). Our current results support investigating anemia status as an allostatic system that responds to infection adaptively, rather than expecting an optimal pre-infection value.

An unexpected trend is the greater influence of paternal grandparents on child anemia status than maternal grandparents. While grandmothers in general play a significant role and influence child nutritional status (Aubel 2012), maternal grandparents have been shown to have a greater effect on child health (Sear et al. 2000) and play a dominant role in deciding what and when the child should eat (Bentley et al. 1999). The observed pattern in our results may reflect a closer relationship with and support from the child's father or a desire to establish social connections with in-laws through child care. Additionally, our study did not establish how far away maternal grandparents lived from children in the study. When interviewing one caregiver about her child's health, the mother mentioned how she often took the child to her mother's home across the street for childcare, even though she resided with her partner's family. Families that live close to both sets of grandparents may have a greater number of caregivers for the child, leading to a greater positive effect on child health.

This study investigates a large range of child, maternal, household, and environmental factors that that allow for an in-depth investigation of predictors for anemia and response to iron supplementation and test more directly the association between children's biology and socio-ecological context. However, the results are limited by several important factors and should be considered preliminary. While the total sample size is relatively large (n = 102), the subsample of children who received iron supplementation is considerably smaller (n=50). This small sample size may have limited power to find statistically significant differences between response and non-response. Additionally, while some studies suggest that Hb may not be affected by iron supplementation and recommend including measures of iron status (Stolzfus et al. 2004a), others report increases in Hb but not in markers of iron status (serum ferritin and free erythrocyte protoporphyrin) in participants receiving daily iron supplementation (Zavaleta et al. 2000). Due to the conflicting reports on iron supplementation's impact on Hb status, future research should explore the effects of iron supplementation on additional biomarkers associated with anemia and iron level.

Despite these limitations, these findings highlight the importance of incorporating a developmental microniche perspective and methodology in research investigating childhood anemia and response to iron supplementation. Factors that affect the prevalence and distribution of anemia in a population involve the complex interplay of political, ecological, social, and biological factors. Given the persistent nature of childhood anemia in Peru and the failure of interventions focusing on the iron supplementation and fortification strategies, understanding the roles of the developmental microniche and factors that can be modified to improve nutritional status and disease exposure is a critical step in reducing childhood anemia prevalence. The associations between child growth patterns coupled with maternal perceptions of child body size, household composition, and seasonal variation with anemia status and response to iron supplementation presented in this paper, indicate the importance of including analysis of caregivers, household, and environmental-level variables in addition to individual-level characteristics in studies of childhood anemia.

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FIGURES

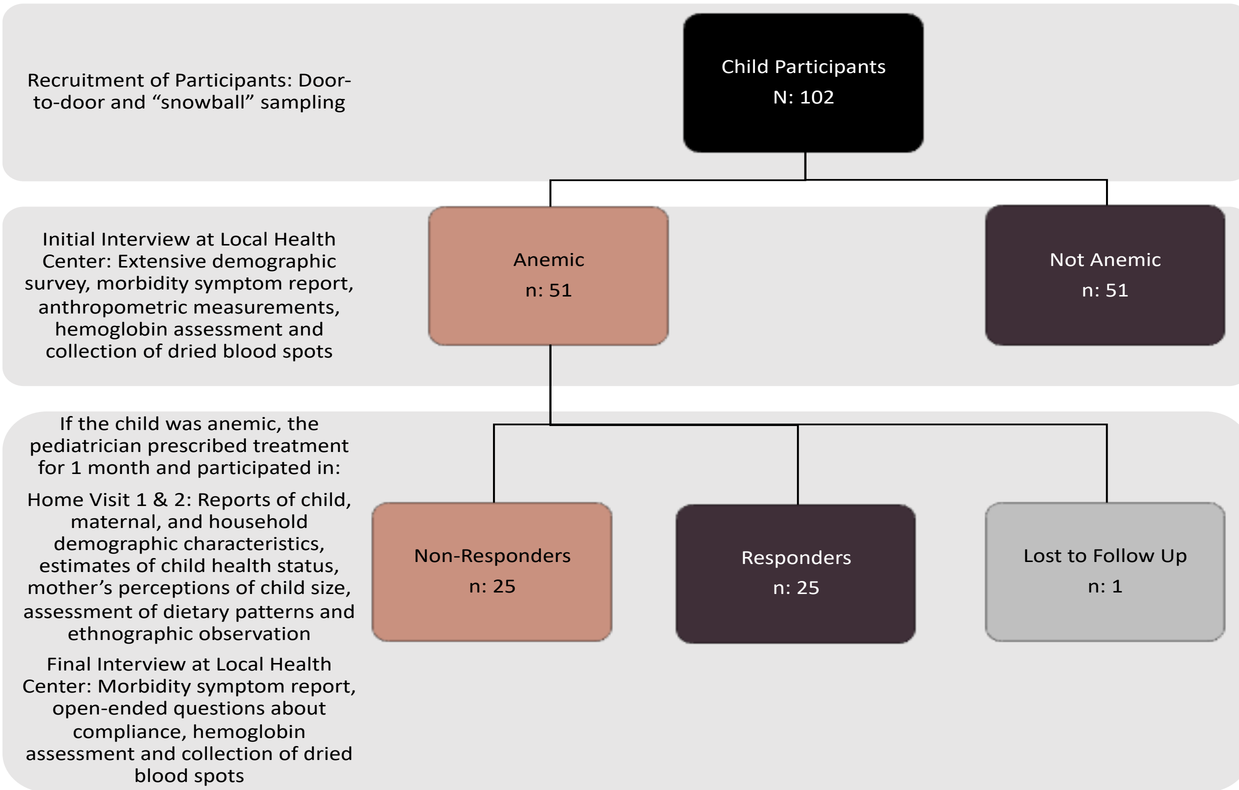
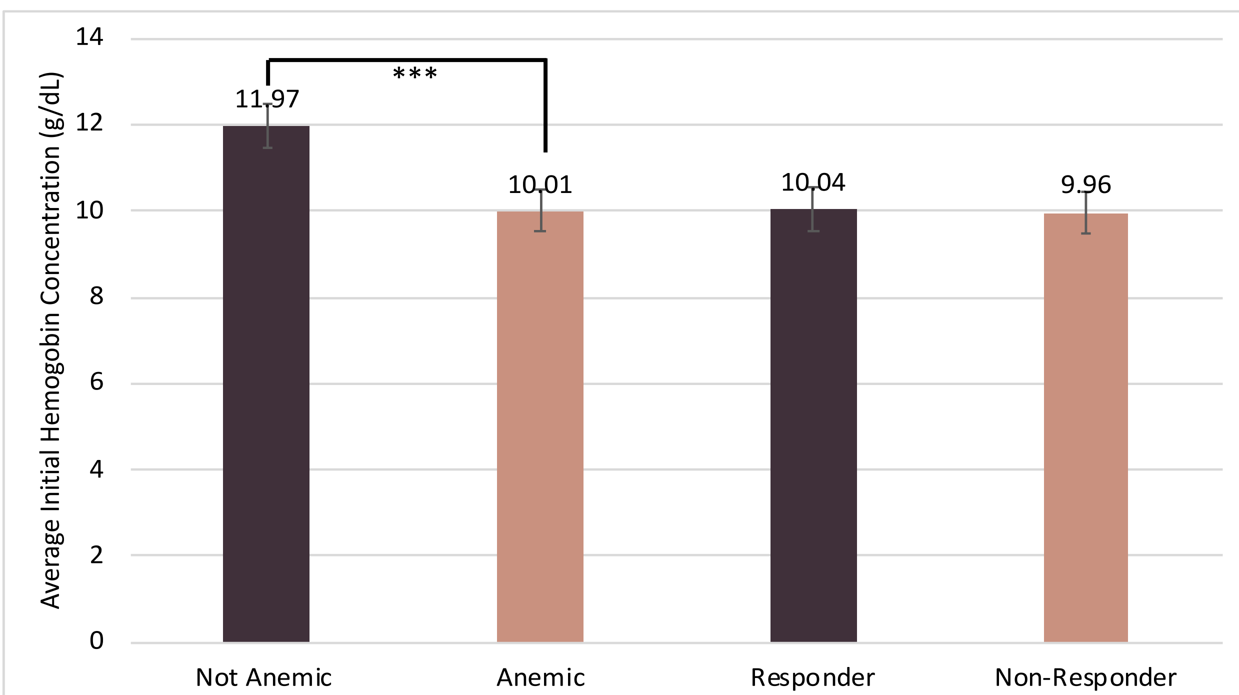


FIGURE 4.1: Study design



***= p-value <0.05

FIGURE 4.2: Mean difference in initial hemoglobin concentration by anemia status and response to iron supplementation

TABLES

TABLE 4.1: Descriptive statistics of child-level variables for anemia status and response to supplementation

Child Descriptives	**Total** N=102	**Not Anemic** n=51	**Anemic** n=51	**Responder** n=25	**Non-Responder** n=25
			Mean (SD) / N (%)		
Hemoglobin Concentration (g/L)	10.99 (1.20)	11.97 (0.69)	10.01 (0.67)	10.04 (0.52)	9.96 (0.81)
Sex (female)	50 (49.02)	26 (50.98)	24 (47.06)	11 (44.0)	12 (48.0)
Age (months)	45.43 (13.8)	49.30 (12.64)	41.57 (13.93)	41.52 (15.26)	42.32 (12.57)
Weight-for-Age Z-Score	-0.07 (1.04)	.17 (0.94)	-0.30 (1.09)	0.02 (1.12)	-0.67 (0.96)
Height-for-Age Z-Score	-0.96 (1.07)	-0.82 (0.90)	-1.10 (1.21)	-0.80 (1.34)	-1.47 (0.98)
Adiposity (>0.5)	82 (80.39)	40 (78.43)	42 (82.35)	23 (92.00)	18 (72.00)
Food Frequency					
Iron Rich: Heme (never)	------	------	------	2 (8.0)	8 (32.0)
Leafy Greens (<1x/week)	------	------	------	8 (32.0)	14 (56.0)
Adherence (>=22 days)	------	------	------	12 (48.0)	14 (56.0)
Initial Interview					
Elevated CRP	20 (19.61)	10 (19.61)	10 (19.61)	3 (12.0)	7 (28.0)
Diarrheal Disease Symptoms	15 (14.71)	3 (5.88)	12 (23.53)	7 (28.0)	5 (20.0)
Cold Symptoms	73 (71.57)	41 (80.39)	32 (62.75)	17 (68.0)	14 (56.0)
Loss of Appetite	46 (45.10)	25 (49.02)	21 (41.18)	12 (48.0)	9 (36.0)
Respiratory Infection Symptoms	15 (14.71)	8 (15.59)	7 (13.73)	4 (16.0)	3 (12.0)
Final Interview					
Elevated CRP	------	------	------	4 (16.0)	10 (40.0)
Diarrheal Disease Symptoms	------	------	------	3 (12.0)	2 (8.0)
Cold Symptoms	------	------	------	14 (56.0)	17 (68.0)
Loss of Appetite	------	------	------	9 (36.0)	9 (36.0)
Respiratory Infection Symptoms	------	------	------	1 (4.0)	2 (8.0)
Parasites (+)	------	------	------	7 (28.0)	9 (36.0)
Bathroom (diaper)	------	------	------	6 (24.0)	10 (40.0)
Consumption of Dirt	------	------	------	11 (44.0)	5 (20.0)

TABLE 4.2: Descriptive statistics of maternal-level variables for anemia status and response to supplementation

Maternal Descriptives	**Total** N=86	**Not Anemic** n=44	**Anemic** n=45	**Responder** n=22	**Non-Responder** n=24
			Mean (SD) / N (%)		
Hemocue Concentration	12.13 (1.55)	11.79 (1.42)	12.51 (1.62)	11.89 (1.22)	11.70 (1.62)
Maternal Age	30.8 (6.75)	30.33 (7.19)	31.25 (6.36)	30.85 (6.94)	30.67 (6.28)
Maternal Education (completed high school)	56 (58.95)	23 (50.0)	33 (67.5)	13 (54.17)	19 (76.0)
Works Outside the Home	40 (42.11)	21 (45.65)	19 (38.78)	11 (45.83)	8 (33.33)
Perceived Child Size (smaller than average)	44 (43.14)	20 (39.22)	24 (47.06)	12 (48.0)	12 (48.0)
Knowledge of Anemia	------	------	------	24 (96.0)	22 (88.0)

TABLE 4.3: Descriptive statistics of household and environmental-level variables for anemia status and response to supplementation

Household Descriptives	**Total** N=76	**Not Anemic** n=45	**Anemic** n=43	**Responder** n=23	**Non-Responder** n=23
			Mean (SD) / N (%)		
Grandmother's First Language (Spanish)	64 (71.1)	30 (68.18)	34 (73.91)	15 (65.22)	18 (81.82)
Monthly Income Range (750-1500 soles)	48 (48.0)	24 (48.0)	24 (48.0)	12 (50.0)	11 (44.0)
Weekly Food Expenditure (soles)	186.67 (64.43)	183.74 (60.31)	189.60 (68.79)	178.33 (60.16)	198.20 (76.57)
Grandparents (paternal)	32 (31.3)	12 (23.5)	20 (39.2)	4(16.0)	7 (28.0)
Number of Children <5 years	2.3 (1.30)	2.12 (1.12)	2.48 (1.45)	2.42 (1.14)	2.6 (1.71)
Persons per Bedroom	------	------	------	2.55 (1.46)	3.40 (1.19)
Pets	------	------	------	16.0 (66.0)	17 (60.0)
Food secure	------	------	------	8 (32.0)	10 (40.0)
Environment Descriptives	N=102	n=51	n=51	n=25	n=25
Trash Disposal (outside)	------	------	------	11 (44.0)	11 (44.0
Season (summer)	59 (57.8)	35 (68.6)	24 (47.1)	12 (48.0)	22(88.0)

TABLE 4.4: Multivariate logistic regression models predicting anemia status for child, maternal, and household and environmental-level models and final model

Variables categorized by level	Level Models OR (CI)	Final Model OR (CI)
CHILD		
Age (months)	***0.96 (0.93-0.99)	0.97 (0.93-1.01)
Weight-for-Age Z-Score	***0.56 (0.36-0.86)	***0.52 (0.31-0.84)
Diarrheal Disease Symptoms (present)	***6.63 (1.47-29.98)	2.78 (0.23-34.12)
Cold Symptoms (present)	**0.38 (0.13-1.15)	0.67 (0.17-2.63)
MATERNAL		
Education (completed high school)	*2.06 (0.74-5.74)	1.75 (0.53-5.80)
Hemoglobin Concentration	0.80 (0.56-1.15)	-----
HOUSEHOLD AND ENVRONMENT		
Grandparents (paternal)	**0.36 (0.13-1.01)	**0.38 (0.12-1.20)
Number of Children <5 years	1.14 (0.78-1.66)	-----
Season (summer)	***3.23 (1.15-9.11)	**2.70 (0.87-8.36)

OR= Odds Ratio, CI=Confidence Interval
*= p-value < 0.20, **= p-value <0.10, ***= p-value <0.05

TABLE 4.5: Multivariate logistic regression models predicting response to iron supplementation for child, maternal, and household and environmental-level models and final model

Variables categorized by level	Level Models OR (CI)	Final Model OR (CI)
CHILD		
Weight-for-Age Z-Score	*2.08 (0.85-5.08)	***2.08 (1.08-4.02)
Height-for-Age Z-Score	0.94 (0.43-2.07)	
Leafy Greens (>=1x/week)	2.32 (0.62-8.66)	
Elevated CRP (initial)	0.75 (0.11-4.93)	
Elevated CRP (final)	*0.32 (0.07-1.41)	***0.07 (0.01-0.67)
MOTHER		
Education (completed high school)	**0.31 (0.08-1.16)	0.46 (0.05-5.18)
HOUSEHOLD AND ENVIRONMENT		
Persons-per-bedroom	***0.64 (0.41-0.98)	0.70 (0.36-1.36)
Season (summer)	***0.15 (0.03-0.69)	***0.04 (0.00-0.37)

OR= Odds Ratio, CI=Confidence Interval
*= p-value < 0.20, **= p-value <0.10, ***= p-value <0.05

CHAPTER 5. ANEMIA, ADIPOSITY, AND PATHOGEN EXPOSURE

Adiposity and pathogen exposure: An investigation of response to iron supplementation in anemic pre-school-aged children living in a dual burden environment

INTRODUCTION

Anemia, or low concentrations of hemoglobin (Hb), is an important risk factor for the health and development of children. Anemia can cause fatigue and low productivity and adversely affects cognitive and motor development (Balarajan et al. 2011; Stoltzfus et al. 2004). Due to concerns about anemia's negative impact on child development and because the majority of anemia cases are attributed to iron deficiency (Stoltzfus et al. 2004), global health institutions recommend iron supplementation and fortification of all children in populations with a high prevalence of anemia (World Health Organization [WHO] 2002; Stolzfus and Dreyfuss 1998). Supplementation and fortification have been proven to be effective public health interventions to reduce anemia rates (Thompson et al. 2013; Zimmermann and Hurrell 2007; Baltussen et al. 2004). A comprehensive review of the efficacy of iron supplementation concluded that the majority of randomized-control-trials investigating the effectiveness of iron supplementation in children report significant increases in Hb concentration and other iron status indicators as well as reduced anemia prevalence (Iannotti et al. 2006).

Despite the reported benefits of iron supplementation, public health officials have raised concerns about the risks associated with iron supplementation among preschoolers. Recent systematic reviews of randomized, controlled trials of iron supplementation conclude that supplementation moderately increases the risk of diarrheal disease (Gera and Sachdev 2002) and malaria (Oppenheimer 2001). Studies documenting the association between iron level and infection caused by malaria parasites with (Sazawal et al. 2006) and without (Nyakeriga et al. 2004) iron supplementation demonstrate the importance of considering rates of infection when designing anemia intervention programs.

One explanation for the negative effects of iron supplementation on health comes from the field of evolutionary medicine, which proposes that some manifestations of disease may act as adaptive defenses against other types of disease (Ewald 1994, Williams and Nesse 1991). Infection with common pathogens has been linked to higher C-reactive protein (CRP), an inflammatory acute-phase protein commonly used as a marker of systemic inflammation, levels in children (Dowd et al. 2010) and adults (Nazmi et al. 2010; Zhu et al. 2000) from the United States, with greater risk of inflammation seen with increasing pathogen burden. Inflammation is one of the first responses of the immune system to infection and signals biological systems to sequester iron, reduce iron absorption and decrease erythropoiesis (the production of red blood cells) which causes a decrease in serum Hb, resulting in anemia (Weinberg 1992). Decreasing the amount of circulating iron and iron absorption restricts the availability of iron to pathogens, inhibiting pathogen growth, proliferation, and virulence (Nemeth and Ganz 2006). In this case, while childhood anemia impairs growth and cognitive development, in areas with high disease ecology anemia may be beneficial as it reduces bacteria proliferation and virulence (Wander et al. 2009).

Research suggests that both chronic pathogen exposure and high levels of adiposity activate pro-inflammatory pathways (McDade et al. 2008a; Vahdat et al. 2012), thus overweight and obesity may also influence the efficacy of iron supplementation programs. In countries undergoing rapid dietary and lifestyle changes, obesity exists alongside illnesses associated with undernutrition, a phenomenon known as the ‘dual burden of disease’ (Popkin, Adair, Ng 2012).

A common manifestation of the dual burden in individuals is the co-occurrence of overweight and anemia. The co-occurrence between the two is attributed to increased iron requirements among overweight individuals (Yanoff et al. 2007) or physiological changes associated with overweight that influence iron absorption and utilization, such as an increase of inflammatory acute-phase proteins, like CRP (Cheng et al. 2012). High levels of these proteins cause inflammation, triggering an innate immune response and increases one's risk for anemia due to iron sequestration and reduced iron absorption.

While the inverse correlation between adiposity and iron-levels was established in the early 1960s (Seltzer et al. 1963; Wenzel et al. 1962), more recent evidence of the association between overweight and anemia has been mixed. Some studies report that higher body mass index (BMI) results in increased risk for iron deficiency and anemia among children and adolescents in both high income and transitioning settings (Aberli et al. 2011; Eftekhari et al. 2009; Zimmermann et al. 2008; Nead et al. 2004). Other studies have observed lower rates of anemia in women and children experiencing overnutrition (Kroker-Lobos et al. 2011; Eckhardt et al. 2008). In a study examining the efficacy of iron supplementation, Baumgartner et al. (2013) report that South African children with high BMI-for-age-z-scores have a greater risk for remaining iron-deficient after iron supplementation for 8.5 months when compared to children with low BMI-for-age-z-scores. These seemingly contradictory findings may be due to a complex set of environmental and individual variables that include differences in disease exposure and immune activation caused by specific economic and cultural contexts. Additionally, while BMI has traditionally been used as a proxy for body fat (Albrecht et al. 2014), other anthropometric measures of over-nutrition, like waist circumference, may provide greater insight into the relationship between adiposity and anemia due to differences in in the production of inflammatory cytokines by fat tissue.

Investigating iron supplementation from an evolutionary medicine perspective within a dual burden context is important to address in children because of their high exposure to pathogens and energetic demands for growth and immune function. Children's mobility and increased independence exposes them to a wide range of pathogens and due to the naivety of the immune system in infancy and childhood, innate immune responses are incredibly important in the defense against infection. While this nonspecific immune response may protect against infection, chronic anemia in childhood can cause decreased cognitive development and physical performance.

Life history theory offers a framework for investigating the benefits and risks of anemia, as energy devoted to immune defense can't be allocated to growth and development (Stearns 1992; Charnov 1993). The trade-off between allocating limited resources to immune defense or growth differs for those living in energy-rich environments versus those living in energy-poor contexts. Children with reliable access to nutrients are able to replenish the costs of immune activation while children experiencing undernutrition have more limited energy to devote to growth and consequently exhibit impaired immunity (McDade et al. 2008b). The simple comparison between energy-rich and energy-poor environments is challenged by a global rise in overnutrition and urbanization, resulting in the dual burden of disease. While fat represents stored energy that is utilized for metabolic processes associated with growth and immune function (Kuzawa 1998), higher body weight and BMI have been associated with chronic inflammation in adults and children (Choi et al. 2013; Dowd et al. 2010; Cook et al. 2000). Additional studies report that the association between central adiposity and pro-inflammatory markers may be particularly strong in younger populations (Fransson et al. 2010; Nguyen et al. 2009). This suggests that high energy stores (in the form of body fat) may not always be beneficial.

Peru provides an important setting to study the efficacy of iron supplementation on child anemia status within a dual burden context. Peru has experienced rapid urbanization and economic growth since 1990, which has created unplanned communities surrounding urban areas. These peri-urban environments were initially the result of policies that favored highland landlords over rural peasants prior to Peru's agricultural reforms however the number of these communities increased dramatically in the in the 1980s and early 1990s when violence in the highlands perpetrated by *Sendero Luminoso* (Shining Path) guerillas and the state special forces drove another influx of migrants to Lima (Theidon 2010, Seligmann 2004). These communities are characterized by poor quality or informal housing, unhealthy living conditions, and poverty, resulting in greater exposure to risk factors and negative health outcomes.

In addition to the rise of unplanned communities, urbanization and economic growth have contributed to a nutrition transition, mainly within urban areas (Chaparro and Estrada 2012).). In Peru, this shift is represented by high rates of undernutrition and overweight and obesity. Between 2010 and 2015, the national prevalence of childhood overweight increased from 6.2%- 6.8%. Recent evidence suggests that communities at sea-level have the highest rates of child overweight (45.5%) when compared to Andean or Amazonian regions (8.9% and 23.0%, respectively) (Santos et al. 2019). The department of Lima, which contains the nation's capital, had one of the highest rates of childhood overweight and obesity compared to Peru's other 23 departments and while rates of obesity have decreased over time, the stabilization of child overweight prevalence reflects a lack of obesity prevention interventions (Torres-Roman et al.

2018). Despite increasing concerns about overnutrition, most recent governmental and non-governmental initiatives have continued to target illnesses associated with undernutrition, specifically anemia. Estimates predict half of all pre-school age children suffer from anemia in Peru (WHO 2009) and, despite the aforementioned interventions, anemia continues to represent a distinct challenge, even for those living near the city of Lima who have greater access to urban infrastructure.

Due to the recent development of obesogenic environments, current efforts to combat anemia and high exposure to disease, unplanned urban communities in Peru provide a prime setting to explore the dual burden using a life history perspective. This study uses data collected from anemic pre-school-aged children (2-5 years old) receiving iron supplementation and living in the peri-urban community of San Juan de Lurigancho to test two hypotheses. First, high immune activation will be associated with a lack of response to iron supplementation after one month of treatment. Second, variation in body fat stores will moderate the association between immune function and response to treatment. We expect a reduced probability of response to iron supplementation in children with immune activation and high adiposity when compared to children with immune activation and low adiposity (Figure 5.1).

METHODS

Study Design

The primary investigator conducted research in three neighborhoods within San Juan de Lurigancho, one of the poorest districts within metropolitan Lima. This district northeast of the city center has been the site of numerous anemia interventions to date. In 2013, 35.7% of children under five living in this district were diagnosed with anemia. Despite the efforts of the Ministry of Health and community-based organizations, this percentage had *increased* to 41.9% in 2014 (Ministerio de Salud 2015). San Juan de Lurigancho also has the highest rates of chronic malnutrition (8.6%) and overweight (11.0%) in pre-school-aged children compared to other low-income districts around Lima (Navarrete Mejía et al. 2016).

Data collection occurred from November 2017 to July 2018, the study included an initial survey and anemia testing and a follow-up interview after one month of prescribed ferrous-sulfate treatment for each participant. Baseline surveys included demographic and socio-economic data, along with anthropometric measurements and dried blood spot samples from finger pricks from anemic pre-school-aged children. If the child was anemic, a physician met with the care-giver child dyad and prescribed treatment, one tablespoon of ferrous-sulfate syrup twice a day for one month. Follow-up survey data, anthropometrics measurements, and a second finger prick blood sample were collected one month later to assess response to iron supplementation.

Complete baseline data were available for 51 children and follow-up Hb data were available for 50, because the family of one participant moved to another distract during data collection. This participant is not included in any of the analyses below. This research protocol received both University of North Carolina at Chapel Hill IRB and *Instituto de Investigación Nutricional* Ethics Board approval for this project. Parental consent and child assent were obtained prior to enrollment in the study.

Data Collection

Primary caregivers provided information on household demographics, reported morbidity symptoms, and season - a dichotomous variable based on the month in which the initial interview took place, summer (December-April) and winter (May-November). Standard procedures (Lohman et al. 1988) were implemented to collect anthropometric measurements of standing height and weight in light clothing and without footwear. Waist circumference was measured with non-elastic tape midway between the lowest rib and the iliac crest. Tricep skinfold thickness (TSF) was measured to the nearest 0.5mm with precision Lange calipers. The primary author used the same procedures and equipment to collect anthropometric data at baseline and again one month later.

At least one drop of free-flowing capillary blood was collected for immediate Hb analysis via a minimally invasive finger prick and a portable photometer Hemocue machine (Hemocue Hb 201+, HemoCue America, California). Following this measurement, at least three drops of blood were collected for laboratory analysis of CRP. The child's finger was cleaned with alcohol and a sterile disposable microlancet was used to deliver a controlled puncture. A drop of whole blood was placed directly onto the Hemocue cuvette and inserted into the Hemocue machine. Hb levels were estimated and recorded within 30 seconds of the finger prick. Additional drops of blood were then collected on standardized filter paper (Whatman #903 Middlesex, UK), dried, and stored frozen at the local health center in San Juan de Lurigancho until they were transported to the Human Biology Laboratory at the University of North Carolina- Chapel Hill. There, the dried blood spot samples were analyzed for CRP using an adapted enzyme-linked immunosorbent assay (ELISA) protocol for R&D Systems: Quantikine Human CRP Immunoassay. Samples were exposed to above-freezing temperatures for less than 24 hours, within the limits necessary to maintain sample integrity for CRP analysis (McDade et al. 2004).

Study Variables

In our study, we used Hb as an objective measure of anemia status. Hb concentration is the most common hematological assessment method used to measure anemia (Chaparro and Suchdev 2019). In Peru, recent initiatives by the Ministry of Health use Hb to assess anemia status in national-level programs at health centers and in schools, incorporating methods used by these initiatives allows for comparison between reported prevalence and rates of anemia in this sample. Additionally, measuring Hb is inexpensive and easy to measure with field-friendly testing. Thus, despite the lack of specificity for establishing nutritional anemias, such as iron status (Balarajan et al. 2011), Hb was used as an objective measure of anemia in this study. Response to iron supplementation is a dichotomous variable based off of the WHO recommendations (2011). Children who became not anemic (Hb>=11.0 g/dL) after 1 month of treatment, the suggested length of iron supplementation by the Peruvian Ministry of Health (Ministerio de Salud 2015), were categorized as responders while children whose Hb remained below 11.0 g/dL at the final test were categorized as non-responders.

This study employed both subjective and objective measures of child immune activation. We collected child morbidity symptoms from caretakers about children in our sample from caretakers for a subjective measure of health to establish current health and collect health histories. Reported child morbidity indices are based on maternal reports of child illness within the two weeks prior to the final interview. The presence and absence of 'common cold' symptoms is based on the cumulative illness burden of runny nose and cough. This study uses CRP as a direct measure of immune activation. CRP is an acute phase protein involved in the innate immune response (Ballou and Kushner 1992), thus the concentration of CRP increases in response to inflammation providing an indicator of immune response that requires energetic resources (Rousham et al. 1998). We use a 2.2 mg/L cut-off value to define children with low versus high concentrations of CRP (Wander et al. 2009; Caminiti et al. 2016).

To measure child adiposity, we calculated and utilized several anthropometric measurements. Waist to height ratio (WHtR) was calculated by dividing the participant's waist circumference by height, if a score was at or above 0.5 the child was categorized as having a high ratio (Ashwell and Hsieh 2005). Measures of BMI and TSF z-scores were calculated based on the WHO standard using the WHO 2007 STATA macro package (de Onis et al. 2007). High and low measures were dichotomized, children with greater than one standard deviation above zero were categorized as having high measures of body fat.

Data Analysis

Investigators first used bivariate logistic regression models to assess the relationships between response to iron supplementation, immune activation, and body size variables. To test the hypothesis that immune activation is associated with reduced ability to respond to iron supplementation, we investigated the association with elevated CRP with changes in Hb concentration over one month of iron supplementation in multivariate logistic regression models including age, sex, and season of data collection. We evaluated a wider range of individual, maternal, household, and environmental variables in previous analyses (Chapter 4), but none approached significance as predictors of response to iron supplementation and are therefore not included as covariates here.

To test the hypothesis that body fat moderates the impact of immune activation on response to iron supplementation, we included several body fat variables in the additional multivariate logistic models. Moderation was tested through the inclusion of interaction terms representing the interaction between immune activation and body fat measures (high WHtR, TSF z-score, and BMI z-score) at the final interview. Statistical analyses were conducted with STATA 13. In all logistic regression models, age, sex, and season were included as covariates and robust standard errors accounting for clustering by maternal identification to account for siblings were used. This process adjusts for siblings included in the sample and provides a more conservative estimate of variance within the models.

RESULTS

Sample characteristics

On average, children's Hb concentration increased by 0.86 g/dL. However, only 50% of children in this sample responded to 1-month of iron supplementation (Table 5.1). More than half of all children in this sample received iron supplementation treatment for 22 days or more during the month of treatment. Children who responded to treatment were slightly older than non-responders and the majority of non-responders participated in the study during the summer months. High WHtR and TSF z-score were common in the sample but a high BMI z-score was not, with only 28% of children having a BMI z-score >1.0. More than half of the sample experienced common cold symptoms two weeks prior to the final interview.

Immune activation and response to supplementation

We hypothesized that high immune activation would be associated with a lack of response to iron supplementation after one month of treatment. We performed logistic regression models controlling for age, sex, and season to test the association between immune activation and response to treatment. Reported cold symptoms were not associated with response to iron supplementation (OR:0.40, CI:0.09-1.77, p-value:0.23) and were therefore excluded from further testing. CRP was associated with response to supplementation in logistic regression models (OR: 0.19, CI: 0.03-1.08, p-value <0.10). Children with high CRP levels were less likely to respond to treatment compared to children with low CRP levels.

Due to the observed differences between reported symptoms and CRP, we tested the relationship between the two variables. Reported cold symptoms were not significantly associated with CRP (OR:1.75, CI:0.41-7.45, p-value:0.45). This lack of association could be due to the fact that CRP concentrations can be elevated in the absence of observable symptoms and individual variation in reporting symptoms (Rousham et al. 1998).

Adiposity as a moderator of immune activation and response to supplementation

We hypothesized that variation in body fat stores will moderate the association between immune function and response to treatment and tested this hypothesis by performing logistic regression models controlling for age, sex, and season. WHtR (OR:32.54, CI: 2.67-396.08, p-value: <0.05) and BMI z-score (OR:3.76, CI:0.96-14.79, p-value: <0.10) were associated with response to supplementation in logistic regression models. TSF z-score (OR:1.04, CI:0.27-4.02, p-value:0.96) was not significantly associated with response to treatment and were therefore not included in further testing.

We found evidence that WHtR moderates the relationship between final CRP and response to supplementation (p-value <0.10) (Table 5.2). Anemic children with low CRP at the end of treatment and high WHtR have the highest predicted probability of responding to supplementation (71%) (Figure 5.2). Preschoolers with low WHtR have a significantly lower chance of responding to iron supplementation than in children with high WHtR, whether they are categorized as having low or high CRP values.

In our sample, BMI z-score moderates the relationship between immune activation and response to ferrous sulfate treatment (p-value <0.05) (Table 5.2). Children with high final immune activation and low BMI z-scores have the lowest predicted probability of recovering from anemia (15%) while participants categorized as having high BMI z-scores with both high and low immune activation after 1-month of treatment have a greater probability of responding to iron supplementation (Figure 5.3). It is interesting to note that children categorized with both low BMI z-score and low CRP values have a significantly higher chance of responding to treatment (55%) than preschoolers with low BMI z-score but an elevated level of CRP (15%).

To explore the different patterns between WHtR and BMI z-score, we tested their relationship to each other. WHtR and BMI z-score were associated with each other (OR:12.23, CI:2.59-57.73, p-value:0.00). However, we found a significant association between BMI z-score and TSF z-score (OR:1.66 CI:1.09-2.55 p-value:0.02) but not between WHtR and TSF z-score (OR:1.53, CI:0.92-2.54, p-value:0.10).

To test the sensitivity of our findings, we explored BMI z-score as a continuous variable in moderation models. We expected a reduced probability of response to iron supplementation in children with high immune activation and adiposity when compared to children with low immune activation and adiposity (Figure 5.1). Our results show a greater difference in the probability of responding to one month of ferrous sulfate syrup between children with low and high immune activation when BMI z-scores fall below average (Figure 5.4). When BMI z-score is higher than 1 standard deviation above average, the chance of responding to treatment is no longer significantly different between children with low and high final CRP. Thus, in our sample, larger body size is associated with response, whether or not children have high immune activation.

DISCUSSION

When iron supplementation has not been effective in reducing anemia individually, experts suggest investigating poor compliance (Galloway and McGuire 1994) and malabsorption (Lopez et al. 2016). Despite more than half of caregivers reporting adhering to the ferrous sulfate syrup regiment for 22 days or more, only half of the children enrolled in this study recovered from anemia at the end of one month of treatment, the standard clinical treatment protocol in Peru. This low rate of response to iron supplementation with high rates of adherence to treatment may demonstrate reduced iron absorption.

We expected an association between higher immune activation and lack of response to iron supplementation. Children who did not respond to treatment had higher rates of common cold symptoms and higher mean CRP. While the presence of cold symptoms was not associated with response to iron supplementation, high CRP reduced a child's odds of responding to iron supplementation. The different patterns observed between our subjective and objective measures of immune activation may be due to individual variation in reporting child morbidity symptoms or that CRP concentrations can be elevated despite a lack of observable symptoms (Panter-Brick 2001; Rousham et al. 1998). These results support previous work exploring child anemia status without iron supplementation that report an association between anemia (with or without iron deficiency) and inflammation (Hadley and DeCaro 2015; Schulze et al. 2014; Wander et al. 2009). Our finding that immune activation is associated with a lack of response to iron supplementation after one month of treatment demonstrates life history theory's potential to provide insight into patterns of anemia.

Rates of overweight in the sample varied depending on adiposity measure, with the highest rates of overweight measured by WHtR and the lowest rates of overweight with BMI z-score. Higher WHtR and BMI z-scores, measures of central adiposity and overall weight for height, respectively, were associated with increased odds of responding to treatment. TSF z-score, a measure of subcutaneous fat, was not associated with response to iron supplementation in preliminary models and was excluded from models exploring our second hypothesis, that variation in body fat stores will moderate the association between immune function and response to treatment suggesting that type of distribution of body fat may be important.

We found that both BMI z-score and WHtR moderate the interaction between CRP and response to iron supplementation, but the two adiposity measures had different probability patterns. Children with a high BMI z-score (in both high and low CRP groups) and those with low BMI z-score and low CRP all had greater than a 50% probability of responding to iron supplementation. Participants with low BMI z-score and high CRP were the least likely to respond to treatment. While a high probability of responding to treatment was expected from children with low CRP and low BMI z-score when compared to children with high CRP and low BMI z-score, the higher probability of responding to treatment among children with high BMI z-scores with either high or low CRP was not.

While we expected a reduced probability of response to iron supplementation associated with high immune activation compared to low immune activation in children with low and high body fat, we observed a different pattern when exploring BMI z-score as a continuous variable. While children with low CRP were more likely to respond to treatment than participants with high CRP, lower BMI z-score was associated with decreased probability of responding to treatment and the odds of responding did increase as BMI z-score approached zero in both CRP categories. The probability of responding continued to increase steadily for those with low CRP and dramatically for those with high CRP.

Generally, higher BMI z-score was associated with a greater likelihood of responding to iron supplementation, whether or not the child had elevated CRP. These results supplement the ambiguous findings in the literature on the relationship between anemia status and BMI (Aberli et al. 2011; Eftekhari et al. 2009; Kroker-Lobos et al. 2011; Eckhardt et al. 2008; Zimmermann et al. 2008; Nead et al. 2004). Oure results support findings from a previous study that reports no relationship between nutritional diagnosis of obesity, overweight, and anemia in a representative sample of children (aged 1015 years) from a rural Lima community and that children in this sample with older age and greater BMI were less likely to present anemia (Rodríguez-Zúñiga 2015). Our findings are counter to previous research on iron supplementation that reported South African children aged 6-11 years with higher BMI z-score were more likely to remain anemic despite receiving treatment than their low BMI z-score counterparts (Baumgartner et al. 2013).

The dissimilarity between our findings and the aforementioned study may be due to differences in sample (e.g. age), study design, diet, and baseline disease ecology. In Peru, where diet quality is likely to be poor (Creed-Kanashiro et al. 2003), the overweight children in this sample may have accrued enough iron or other minerals related to iron absorption (e.g. vitamin A and zinc) that lower their risk of anemia compared to non-overweight participants (Eckhardt et al. 2008).

While previous studies exploring the relationship between weight and anemia status used BMI to represent adiposity, we expanded our investigation to include an additional measure of body fat, WHtR. This variable was investigated due to the disproportionate increase in waist circumference relative to overall body mass in in Mexico and China, two middle-income countries undergoing rapid economic and urbanization transitions similar to the shifts observed in Peru (Albrecht et al. 2015). We found that higher WHtR was associated with a greater probability of responding to treatment, but the likelihood of responding to treatment in the WHtR categories differed from the corresponding BMI z-score categories. Children with high WHtR and low CRP had the greatest probability of responding to treatment compared to children with high WHtR and high CRP and children with low WHtR. Participants with low WHtR are the least likely to respond to treatment, despite having high or low CRP.

The variation in patterns observed between the two adiposity measures may be due to differences in what the body fat variables were measuring. In this sample, BMI z-score was associated with TSF z-score, WHtR was not. This suggests that BMI is a better measure of peripheral body fat while WHtR may better reflect an accumulation of visceral adipose tissue. Visceral adipose tissue produces a variety of pro-inflammatory cytokines that stimulate CRP production, such as IL-6 and TNF-α (Thompson et al. 2015). Elevated pro-inflammatory cytokines in children with greater central body fat may explain the lower probability of responding to treatment in children with high WHtR and CRP when compared to children with high BMI z-score and CRP.

The cumulative effect of high CRP and other pro-inflammatory cytokines may also explain the variation in probability of responding to treatment between children with high and low CRP among participants with high WHtR. Having high CRP along with high levels of other pro-inflammatory markers may work together to reduce a child's probability of responding to treatment due to a stronger innate immune response (resulting in iron sequestration and/or lack of iron absorption), while having low CRP doesn't create a larger cumulative effect. Research investigating the environmental and behavioral risk factors associated with central obesity and inflammation in Chinese adults report evidence for this potential association. Thompson et al (2015) found that men and women with high WHtR and inflammation were more likely to have infectious disease symptoms than those with high WHtR and no inflammation. More work is needed to understand the range of variation in inflammatory processes associated with central and peripheral adiposity.

Our results are limited by several important factors, the sample of anemic children who received iron supplementation is small (n=50). This small sample size resulted in large confidence intervals in logistic regression models and may have limited power to find statistically significant differences between responders and non-responders. Despite Hb being an inexpensive and easy to measure with field-friendly testing, it lacks specificity for establishing nutritional anemias, such as iron status. Additionally, while some studies suggest that Hb may not be affected by iron supplementation and recommend including measures of iron status (Stolzfus et al. 2004), others report increases in Hb but not in markers of iron status (serum ferritin and free erythrocyte protoporphyrin) in participants receiving daily iron supplementation

(Zavaleta et al. 2000). Due to the conflicting reports on iron supplementation's impact on Hb status, future research should explore the effects of iron supplementation on additional biomarkers associated with anemia and iron level. Another limitation of our study is the use of a single inflammation measure, CRP. To further explore the association between adiposity, immune activation, and response to iron supplementation a variety of pro-inflammatory cytokine biomarkers, such as IL-6 and TNF-α, should be used in future research.

Despite these limitations, these results further our understanding of the relationship between immune activation and anemia status within a dual burden context. This study demonstrates evolutionary medicine's potential to provide insight into patterns of disease and highlights the need for further investigation of child inflammatory profiles within a dual burden context. The human immune system is characterized by substantial developmental plasticity. Longitudinal research on immune function demonstrates nutritional and microbial exposures in early childhood are important determinants of inflammation in adulthood (McDade 2012). The inclusion of central and peripheral adiposity measures in this study expands our knowledge on the influence of visceral adipose tissue on the relationship between immune function and anemia in children. Further research on the variation of inflammatory processes associated with visceral adiposity in childhood is needed to investigate pathways to health and disease later in life.

This research has important public health implications. While the probability of anemia and overweight co-occurring may be low, both of these conditions are caused by malnutrition and have links to chronic disease and negative developmental effects (Stoltzfus et al. 2004; Popkin et al. 2006). The high prevalence of anemia and the rising rates of overweight and obesity in Peru warrant prevention and education efforts as well as further investigation into the dual burden of disease.

CONCLUSION

We found that high immune activation is associated with a lack of response to iron supplementation after one month of treatment and that body fat moderates the association between immune function and response to treatment. Different adiposity measures provide variation in the probability of anemic children responding to iron supplementation treatment. Children with low CRP and high WHtR are the most likely to respond to treatment when compared to participants with low WHtR and high WHtR with low CRP. In contrast, children with high BMI z-score (with both high and low CRP) as well as low BMI z-score and CRP had greater odds of responding to treatment than children with low BMI z-score and high CRP. While we expected a reduced probability of response to iron supplementation associated with high immune activation compared to low immune activation in children with low and high body fat, this pattern did not remain with BMI z-scores greater than zero.

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FIGURES

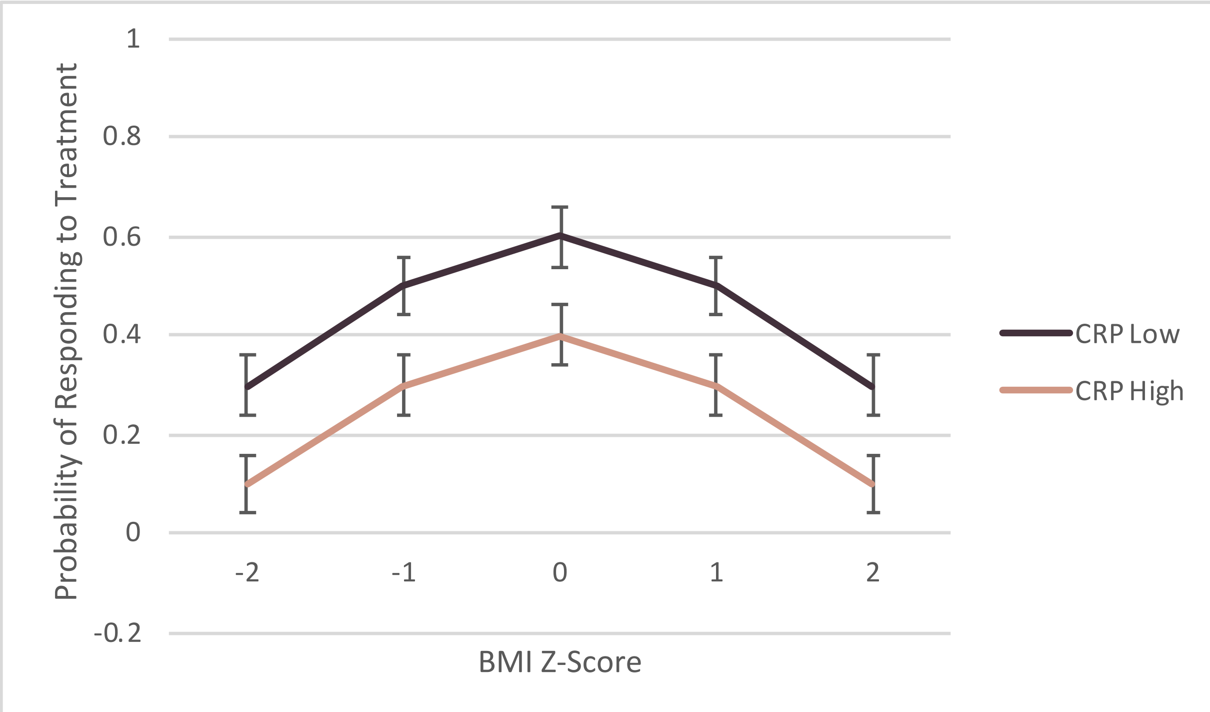
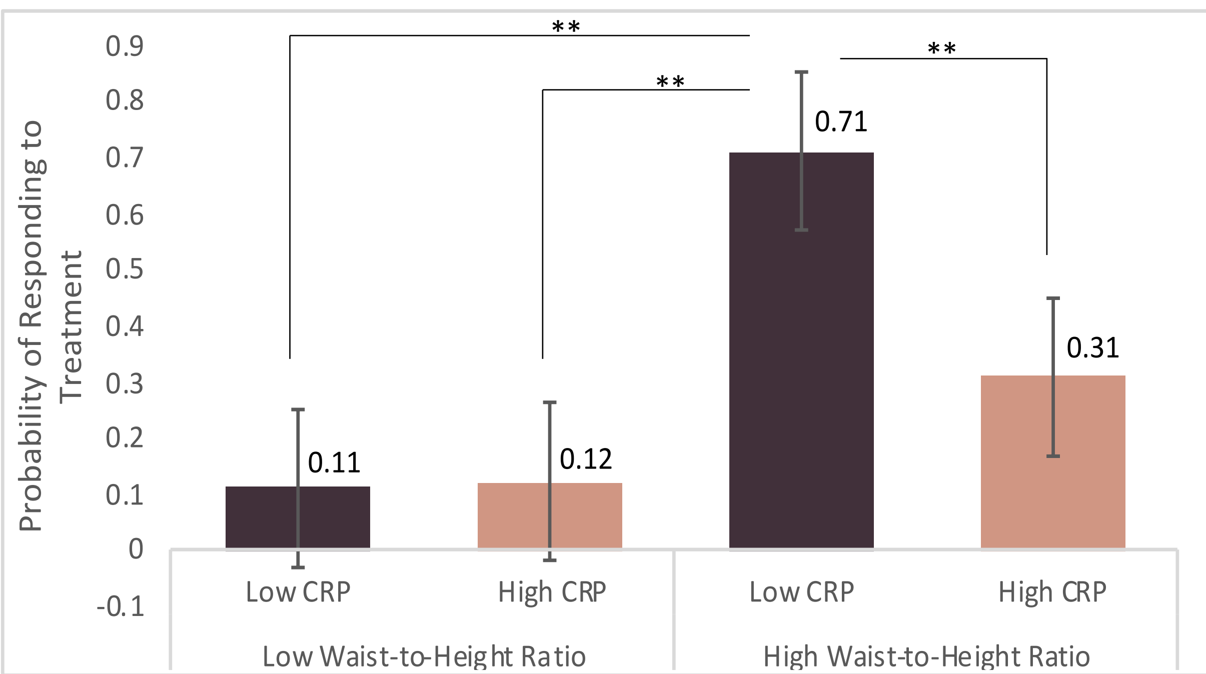
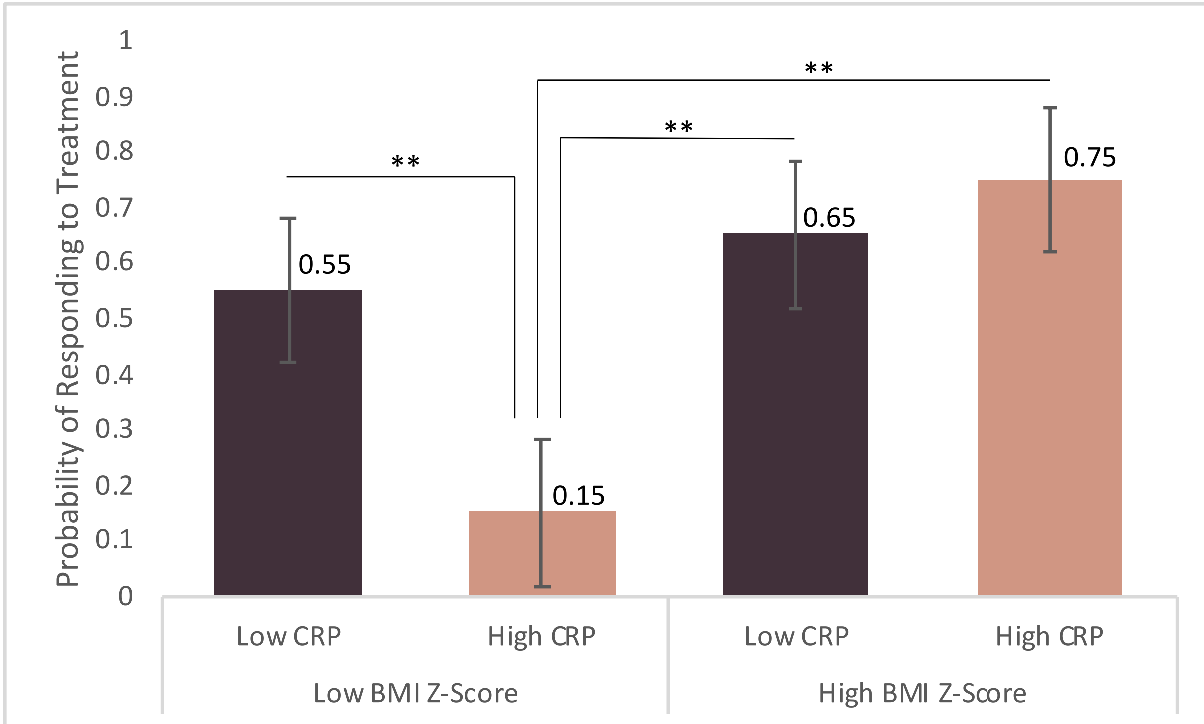


FIGURE 5.1: Hypothesized probability of responding to treatment by BMI Z-score and categorized by low and high CRP



**p-value <0.05

FIGURE 5.2: Probability of responding to treatment by and high waist to height ratio and low and high CRP



**p-value <0.05

FIGURE 5.3: Probability of responding to treatment by low and high BMI Z-score and low and high CRP

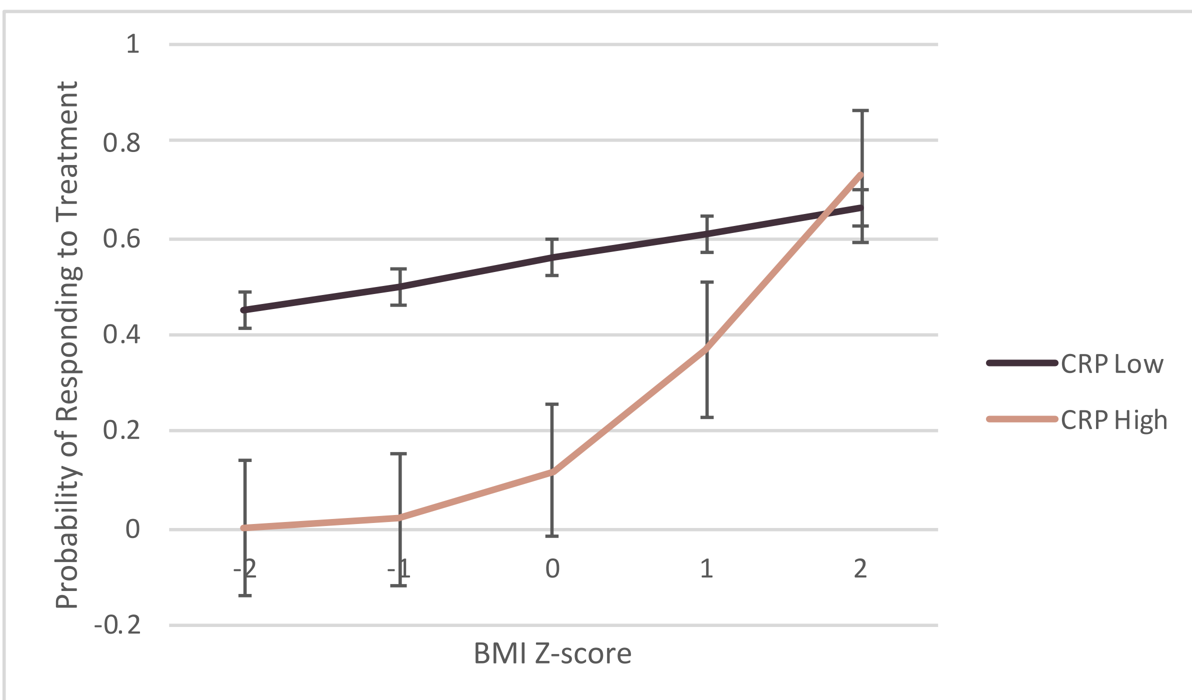


FIGURE 5.4: Probability of responding to treatment by BMI Z-score and categorized by low and high CRP

TABLES

TABLE 5.1: Descriptive characteristics by response to treatment (mean and [SD] for continuous variables)

Variable	Responders	Non-Responders
n	25	25
Initial Hemoglobin (g/dL)	10.04 (0.51)	9.96 (0.80)
Final Hemoglobin (g/dL)	11.60 (0.58)	10.14 (0.63)
Female	11 (44.0)	12 (48.0)
Age (years)	3.04 (1.17)	2.96 (1.02)
Season (summer)	12 (48.0)	22(88.0)
Adherence (>=22 days)	12 (48.0)	14 (56.0)
Presence of Cold Symptoms	14 (56.0)	17 (68.0)
CRP	1.47 (2.02)	2.74 (3.22)
CRP >2.2 mg/L	5 (20.0)	10 (40.0)
Waist to Height Ratio	0.55 (0.04)	0.54 (0.09)
Waist to Height Ratio (>0.5)	23 (92.00)	18 (72.00)
Tricep Skinfold Z-Score	1.52 (1.11)	1.19 (1.14)
Tricep Skinfold Z-Score >1.0	18 (72)	16 (64)
Body Mass Index Z-Score	0.78 (1.02)	0.40 (0.94)
Body Mass Index Z-Score (>1.0)	9 (36.0)	5 (20.0)

TABLE 5.2: Multivariate logistic regression models predicting response to iron supplementation by categorical waist to height ratio and body mass index z-score variables

	Model 1		Model 2	
Variable	OR	CI	OR	CI
Age	*2.23	0.89-5.63	1.28	0.68-2.40
Sex	3.19	0.65-15.52	0.78	0.17-3.50
Season	**0.03	0.00-0.18	**0.04	0.01-0.38
Final CRP >2.2 mg/L	**1.21	0.09-16.05	0.05	0.01-0.50
Waist to Height Ratio (> 0.5)	149.417	8.30-2690.00	---	---
Waist to Height Ratio (> 0.5) X CRP	*0.05	0.00-1.68	---	---
BMI Z-Score (>1.0)	---	---	**1.80	0.35-9.29
BMI Z-Score (>1.0) X CRP	---	---	**34.75	1.08-1120.51

OR= Odds Ratios, CI = Confidence Interval

*p-value <0.10, **p-value <0.05

CHAPTER 6. INTESTINAL MICROBIOTA AND RECOVERY FROM ANEMIA

Iron regulation and response: The role of iron availability and intestinal microbiota diversity on recovery from childhood anemia

INTRODUCTION

Recent advances in technology have allowed for more in-depth investigations of microbes living in or on human hosts. This collection of organisms and their genomes is known as the human microbiome (Qin et al. 2010). Changes in microbial communities have been implicated in the cause of several chronic conditions, specifically with the trillions of micro-organisms living in the human gut (Wu et al. 2013; Larsen et al. 2010; Manichanh et al. 2006). Evidence has also demonstrated links between taxonomic composition of the gut microbiota and malnutrition.

Anemia, a mild to moderate decrease in serum hemoglobin (Hb) most often attributed to iron deficiency (McLean et al. 2007), is especially important to investigate when exploring the intestinal microbiome because of its documented association with both under-nutrition (Gleason and Scrimshaw 2007) and over-nutrition (Aberli et al. 2011; Eftekhari et al. 2009). The intestinal microbiome may also play a key role in iron absorption through microbial metabolism (Petry et al. 2012; Tako et al. 2008) and alterations in intestinal pH level (Salovaara et al. 2003). However, some micro-organisms do not establish a symbiotic relationship with humans when iron is introduced to the gut. While iron is an essential metal for humans it is also vital for the growth and proliferation of many pathogenic bacteria.

Generally, iron in the human body is bound to proteins which limits iron availability to potential infective agents. During infection, the innate immune response sharply reduces iron absorption and sequesters iron to further decrease bioavailability (Oppenheimer 2001). However, this immune defense sequence leaves iron in the gut. In the duodenum and upper jejunum, bacteria compete for unabsorbed dietary iron because the availability of and ability to acquire this vital metal is essential to bacterial colonization, and, for most enteric gram-negative bacteria, also plays an essential role in virulence (Naikare et al. 2006). The immune response against pathogenic bacteria is limited in the intestine as there is no system for the sequestration of iron in the gut lumen (Andrews et al. 2003). While recent studies have not revealed significant differences in phylogenetic diversity between anemic and non-anemic infants and children, there are documented differences in taxa abundance. Jaeggi et al (2015) found that anemic Kenyan infants had lower abundances of *Prevotell*a and higher abundances of *Actinomycetales* and *Streptococcus*. In a study with Kenyan children, better iron status was shown to predict lower amounts of *Escherichia coli* (Paganini et al. 2016).

Despite fortification being an effective strategy to reduce anemia rates (Zimmermann and Hurrell 2007; Baltussen et al. 2004), iron supplementation may lead to a greater concentration of unabsorbed iron in the gut (Kortman et al. 2014). This creates an environment that favors increased colonization of pathogenic bacteria over barrier or protective bacteria, thus generating gut microbiota disequilibrium and increasing morbidity. A comprehensive review of the effects of iron fortification on morbidity in infants and children concluded that that iron supplementation does not significantly increase the risk of overall infection but may increase the risk of developing diarrhea (Gera and Suchdev 2002). More recent studies demonstrate the negative effects of iron fortification on childhood morbidity rates and gut microbiota composition.

Research in Côte d'Ivoire found that children receiving iron fortified wheat flour had an increase in the number of enterobacteria and iron deficient children had unfavorable ratios of fecal enterobacteria to bifidobacteria and lactobacilli at baseline, which only increased with iron fortification (Zimmermann et al. 2010). Jaeggi et al. (2015) demonstrated that the provision of micronutrient powders (MNP) to weaning infants increased the prevalence of Enterobacteria and *Clostridium* and the enterobacteria/bifidobacteria ratio in Kenya. This work provides valuable insight on the gut microbiome as a pathway between iron fortification and morbidity, however its geographic scope is limited to sub-Saharan Africa, ignoring other areas of the world suffering from high anemia prevalence.

One explanation for the negative effects of iron supplementation on health comes from the field of evolutionary medicine, which proposes that some manifestations of disease may act as adaptive defenses against other types of infection (Ewald 1994, Williams and Nesse 1991). In this case, while childhood anemia impairs growth and cognitive development, in areas with high disease ecology anemia may be beneficial as it reduces bacteria proliferation and virulence. Determining when anemia is a disorder or defense is critical to creating appropriate intervention strategies. Establishing the effectiveness of iron supplements and investigating the association between pathogenic exposure, gut microbiota dysbiosis, and anemia is an important step in developing fortification programs.

Factors that affect child pathogen exposure, nutritional status, and the composition of intestinal microbiota involve the complex interplay of political, ecological, social, and biological factors. The concept of the developmental microniche (Super and Harkness 2002; Worthman 1994) is a useful model for exploring the relationship between socioecological context and health. The niche is defined as the variable individual context of each child, and includes the social as well as physical settings in which each child develops (Harkness and Super 1986; Worthman 2010). The niche provides a framework to organize and explore relationships between children's biology and their socio-ecological context (e.g., Brewis 2003). The developmental microniche, therefore, serves as a useful guide for methods and analyses to establish individual disease exposure and how disease ecology can be linked to gut microbiota composition and child response to iron supplementation, creating a more holistic picture in which to investigate evolutionary theories (Figure 6.1).

This study uses an evolutionary medicine perspective to explore the role of increased iron availability via iron supplementation and intestinal microbiota diversity on recovery from childhood anemia in Peruvian pre-school-aged children. We test the gut microbiome as a hypothesized pathway that may link iron supplementation and child recovery from anemia. This study asks: Does one month of treatment with iron supplementation significantly alter the diversity and composition of the gut microbiome? Do baseline and post-treatment gut microbiota diversity and predominant taxa differ in children who respond and don't respond to iron supplementation? Do these differences persist after controlling for relevant pathogenic exposures?

SAMPLE AND METHODS

Setting

Peru suffers from high anemia rates similar to most sub-Saharan African countries (Alcázar 2013). The WHO (2009) has categorized Peru as having "severe" anemia prevalence and estimates that about half of all pre-school age children suffer from anemia. The prevalence of anemia in children under five is higher than the prevalence of malnutrition and has remained constant despite decreases in rates of stunting and poverty (Marini et al. 2017). The high rates of anemia in Peru have been subject to considerable governmental and non-governmental attention and several recent initiatives have aimed to reduce levels of anemia, specifically among children five years and younger. Despite these interventions for reducing anemia and malnutrition more broadly, anemia continues to represent a distinct challenge.

Lima is the largest city in Peru, located on the Peruvian coastal plain. The capital city is considered the political, cultural, and financial center of the country. This results in substantial internal migration to Lima occurs, leading to an annual growth rate of 1.57% (INEI 2013). One outcome of increased population size is the expansion of peri-urban communities in Lima. These communities are characterized by informal or poor-quality housing, unhealthy living conditions, and poverty, resulting in greater exposure to risk factors and negative health outcomes.

San Juan de Lurigancho is a peri-urban district northeast of Lima, which has been the site of numerous anemia interventions to date. In 2013, 35.7% of children under five living in this district were diagnosed with anemia; in 2014, despite the efforts of the Ministry of Health and community-based organizations, the percentage had *increased* to 41.9% (Ministerio de Salud 2015). The lack of success among programs that seek to combat anemia and the expressed concern about anemia from community members also provides an ideal context for investigating iron supplementation in children.

Study Design

The study population is represented by 50 anemic children (23 girls and 27 boys), ages 2-5 years, living in three neighborhoods of San Juan de Lurigancho. This sample represents a relatively disadvantaged area of the Lima district. Research in this community was conducted in collaboration with researchers at the *Instituto de Investigación Nutricional* (IIN), a private, non-profit institution dedicated to interdisciplinary research on health and nutrition in Peru.

Data collection occurred from November 2017 to July 2018. All children included in this study had not consumed antibiotics for the two weeks prior to enrollment. The study included an initial survey and anemia testing and a follow-up interview after one month of prescribed treatment. Baseline surveys included demographic and socio-economic data, along with anthropometric measurements, and the collection of dried blood spots samples. If a child was anemic, the care-giver-child dyad met with a physician and was prescribed treatment, ferrous sulfate syrup two times a day for four weeks. A stool sample was collected before beginning treatment. Follow-up survey data, anthropometrics measurements, and a second finger prick blood sample and stool sample were collected one month later to assess response to iron supplementation.

Complete baseline data were available for 51 children and follow-up Hb data were available for 50, because the family of one participant moved to another distract during data collection. This participant is not included in any of the analyses below. Of the children included in analysis, one did not provide a post-supplementation stool sample. This research protocol received both University of North Carolina at Chapel Hill IRB (#17-0404) and *Instituto de Investigación Nutricional* Ethics Board (#373-2017.P-80) approval for this project. Parental consent and child assent were obtained prior to enrollment in the study.

Response to Iron Supplementation

At the conclusion of the initial and final interviews, the child's capillary Hb was measured to test for anemia via a minimally invasive finger prick and a portable photometer Hemocue machine (Hemocue Hb 201+, HemoCue America, California). The child's finger was cleaned with alcohol, and a sterile disposable microlancet was used to deliver a controlled puncture. Whole blood was placed directly onto the Hemocue cuvette and inserted into the Hemocue machine. The Hb measurement was conducted within 30 seconds of the finger prick. In the following analyses, response to iron supplementation is used as a dichotomous variable, responders are children who became not anemic (>=11.0 g/dL) after 1 month of treatment while non-responders are children whose Hb level remained below 11.0 g/dL at the final test. These categories are based off of the WHO recommendations (2011), and despite the fact that these category boundaries have not changed since 1968, the cut-off points continue to be used in a number of studies and publications.

Pathogenic Exposure

Several variables were used to represent pathogen exposure in this study. CRP is used as a direct measure of immune activation. CRP is an acute phase protein involved in the innate immune response (Ballou and Kushner 1992), thus the concentration of CRP increases in response to inflammation providing an indicator of immune response (Rousham et al. 1998). We use a 2.2 mg/L cut-off value to define children with low versus high concentrations of CRP (Caminiti et al. 2016; Wander et al. 2009). Age, sex, reported consumption of dirt as well as season of the initial interview were also included as these they both indicate differential contact with environmental and pre-school-related disease ecology and are associated with child response to supplementation (Chapter 4). Two distinct seasons can be identified in Lima: summer, from December through April; and winter from May through November. BMI zscore was also included in models as higher levels of body fat are associated with inflammation and immune response (Cheng et al. 2012) and gut bacterial composition and ratios (Ley et al. 2005). BMI z-scores were converted based on the WHO standard using the WHO 2007 STATA macro package (de Onis et al. 2007). High and low measures were dichotomized, children with greater than one standard deviation above zero were categorized as having high measures of body fat. Additionally, anemic children were invited to have parasite tests completed by the local health center, only 18 children opted to complete parasitic testing. If the samples included evidence of parasitic infection, including *Enterobius vermicularis* (human pinworms), a parasite that can contribute to anemia, then the child was labeled positive for parasites.

Gut Microbiota – Sample Collection

If a child was anemic, mothers were asked to collect a small stool sample and store it in the provided containers. If the household had a working freezer the samples were stored there until the next morning when the first author retrieved the sample (typically less than 24 hours). If the household did not have a working freezer, the mothers were asked to bring the sample to the community health center immediately upon collecting the sample. The stool samples were stored in a -25°C freezer at the health center until they were transported to the University of North Carolina – Chapel Hill by the first author at the completion of fieldwork.

Gut Microbiota – DNA Isolation

Samples were transferred to a 2 ml tube containing 200 mg of ≤106 μm glass beads (Sigma, St. Louis, MO) and 0.3 ml of Qiagen ATL buffer (Valencia, CA), supplemented with 20 mg/ml lysozyme (Thermo Fisher Scientific, Grand Island, NY). The suspension was incubated at 37°C for 1 hour with occasional agitation. Subsequently the suspension was supplemented with 600IU of Qiagen proteinase K and incubated at 60°C for 1 hour. Finally, 0.3 ml of Qiagen AL buffer was added and a final incubation at 70°C for 10 minutes was carried out. Bead beating was then employed for 3 minutes in a Qiagen TissueLyser II at 30Hz. After a brief centrifugation, supernatants were aspirated and transferred to a new tube containing 0.3 ml of ethanol. DNA was purified using a standard on-column purification method with Qiagen buffers AW1 and AW2 as washing agents, and eluted in 10mM Tris (pH 8.0).

Gut Microbiota – 16S rRNA Amplicon Sequencing

12.5 ng of total DNA were amplified using universal primers targeting the V4 region of the bacterial 16S rRNA gene1, 2. Primer sequences contained overhang adapters appended to the 5' end of each primer for compatibility with Illumina sequencing platform. The complete sequences of the primers were:

515F - 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA 3'

806R - 5’GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACHVGGGTWTCTAAT 3’

Master mixes contained 12.5 ng of total DNA, 0.2 μM of each primer and 2x KAPA HiFi HotStart ReadyMix (KAPA Biosystems, Wilmington, MA). The thermal profile for the amplification of each sample had an initial denaturing step at 95°C for 3 minutes, followed by a cycling of denaturing of 95°C for 30 seconds, annealing at 55°C for 30 seconds and a 30 second extension at 72°C (25 cycles), a 5 minutes extension at 72°C and a final hold at 4°C. Each 16S amplicon was purified using the AMPure XP reagent (Beckman Coulter, Indianapolis, IN). In the next step each sample was amplified using a limited cycle PCR program, adding Illumina sequencing adapters and dual-index barcodes (index 1(i7) and index 2(i5)) (Illumina, San Diego, CA) to the amplicon target. The thermal profile for the amplification of each sample had an initial denaturing step at 95°C for 3 minutes, followed by a denaturing cycle of 95°C for 30 seconds, annealing at 55°C for 30 seconds and a 30 second extension at 72°C (8 cycles), a 5 minutes extension at 72°C and a final hold at 4°C. The final libraries were again purified using the AMPure XP reagent (Beckman Coulter), quantified and normalized prior to pooling. The DNA library pool was then denatured with NaOH, diluted with hybridization buffer and heat denatured before loading on the MiSeq reagent cartridge (Illumina) and on the MiSeq instrument (Illumina). Automated cluster generation and paired–end sequencing with dual reads were performed according to the manufacturer’s instructions.

Gut Microbiota – Sequencing Data Analysis

Sequencing output from the Illumina MiSeq platform were converted to fastq format and demultiplexed using Illumina Bcl2Fastq 2.18.0.12. The resulting paired-end reads were processed using QIIME 23 2018.11. Index and linker primer sequences were trimmed using the QIIME 2 invocation of cutadapt. The resulting paired-end reads were processed with DADA2 through QIIME 2 including merging paired ends, quality filtering, error correction, and chimera detection. Amplicon sequencing units from DADA2 were assigned taxonomic identifiers with respect to Green Genes release 13_08. Alpha diversity with respect to: Faith PD whole tree, Evenness (Shannon) index, and observed species number metrics; was estimated using QIIME 2 at a rarefaction depth of 5,000 sequences per subsample. Beta diversity estimates were calculated within QIIME 2 using weighted and unweighted Unifrac distances as well as Bray-Curtis dissimilarity between samples at a subsampling depth of 5,000. Results were summarized, visualized through principal coordinate analysis, and significance was estimated as implemented in QIIME 2. Significance of differential abundance was estimated using AnCom as implemented in QIIME 2.

Analytic Methods

We tested for differences in alpha and beta diversity between pre and post samples as well as within both timing groups by response to supplementation using QIIME 2. Alpha diversity measures were calculated using Faith PD whole tree, Evenness (Shannon) index, and observed species number metrics; was estimated using QIIME 2 at a rarefaction depth of 5,000 sequences per subsample. Beta diversity estimates were also calculated within QIIME 2 using weighted and unweighted Unifrac distances as well as Bray-Curtis dissimilarity between samples at a subsampling depth of 5,000.

We also tested for variation in taxa abundance using regression models for each of the aforementioned pairings. Initially these models included just the taxa in question and timing or response variables. For taxa with an abundance greater than 1% and showing evidence of difference (p-value<0.25 was used due to small sample size), we added pathogenic exposure variables to the model to test whether those differences persisted. We focused analysis on taxa abundance at the phyla and order level for taxa having >1% abundance but mention taxa significant in bivariate models here because it is unknown whether these low bacterial abundances would produce a biological impact (McClorry et al. 2018). Additional descriptive statistics and bivariate tests (chi-square tests for categorical variables and T tests for continuous variables) were employed to further explore the relationship between taxa and other variables of interest. All models were adjusted for siblings in the samples by clustering by maternal identification number. All analyses were completed using Stata 13.

RESULTS

While adherence was high, only half of the participating children responded to iron supplementation treatment (Table 6.1). One average, responders were older, had lower CRP, and higher BMI z-scores. A greater number of responders participated in the study during the summer and were reported to have consumed dirt in the past week. Three of the children in this sample had indications of parasitic infection and tested positive for *Enterobius vermicularis*.

Comparison of gut microbiota between pre- and post-treatment samples

Our first research question explored whether one month of treatment with iron supplementation significantly alters the diversity and composition of the gut microbiome. To test this, we examined differences in diversity and phylogenetic diversity. No differences in diversity were observed between pre and post supplementation. We also examined the differences in relative abundance for specific taxa at the phyla (Figure 6.2) and order levels. Post-supplementation samples had a higher abundance of three phyla than pre-supplementation samples (p-value <0.25) but all taxa were under 1% in relative abundance (Table 6.2).

At the order level, the relative abundance of Enterobacteriales, a member of the Proteobacteria phyla, was higher in pre-supplementation samples than in post-supplementation stool samples (p-value = 0.12). This pattern persisted after the inclusion of pathogen exposure variables (p-value = 0.10). Bivariate testing revealed an association between a higher relative abundance of Enterobacteriales and the consumption of dirt (p-value = 0.03) and the summer season (p-value = 0.08). There were a number of other differences observed between pre- and post- supplementation samples (p-value<0.25), but none of the relative abundances were greater than 1% (Table 6.3).

We conducted further testing to explore the relative abundance of taxa at the family and genus levels and ratios of different order taxa to each other that was guided by findings from other studies investigating anemia and the intestinal microbiome (McClorry et al. 2018, Muleviciene et al. 2018). However, models did not show any statistically significant differences in taxa at either level between pre and post supplementation samples.

Baseline gut microbiota and recovery from anemia

The same statistical methods were applied to test whether pre-supplementation gut microbiota diversity and predominant taxa differ between children who respond and don't respond to iron supplementation. No differences in diversity were observed by response within baseline samples. Before treatment, responders had a higher abundance of Spirochaetes (p-value = 0.10) than non-responders, but this phylum was <1% in abundance. Similarly, there were a number of order-level differences between responders and non-responders within samples collected before treatment (p-value <0.10), however all taxa had a relative abundance less than 1% (Table 6.3).

In bivariate models testing differences at the genus and family level, only one family-level taxa, Barnesiellaceae, was significantly higher in children who did not respond to treatment compared to responders in baseline samples (p-value = 0.10). In models controlling for pathogenic exposures, Barnesiellaceae remained significantly associated with response to iron supplementation (p-value = 0.03) (Table 6.5).

Post-treatment gut microbiota and recovery from anemia

Following the same analysis protocol, we investigated whether post-treatment gut microbiota diversity and predominant taxa differ in children who respond and don't respond to iron supplementation. No differences in diversity were observed by response within post-treatment samples. After treatment, responders had higher relative abundance of four different taxa (p-value <0.20) and lower numbers of two phyla (p-value <0.25) (Table 6.2). All phyla were below 1% relative abundance except for Proteobacteria (p-value =0.18).

In models controlling for pathogenic exposures, children who responded to iron supplementation had a lower abundance of Proteobacteria than children who did not respond to treatment (p-value = 0.06) (Table 6.4, Figure 6.3). Bivariate testing between relative abundance of Proteobacteria and co-variates demonstrated an association between higher relative abundance of this taxa and eating dirt (p-value = 0.06) and the summer season (p-value =0.09).

In post-supplementation samples, several order-level differences with an abundance greater than 1% were identified. Enterobacteriales and Lactobacillales had a greater relative abundance in children who did not respond to iron supplementation than in responders (p-value <0.10). Additionally, preschoolers who recovered from anemia had a greater relative abundance of Clostridiales and Bifidobacteriales (p-value <0.20). Several other taxa were found to be higher in responders and non-responders (p-value <0.20), but their relative abundance was less than 1% (Table 6.3).

In models controlling for pathogenic exposures, the relative abundance of Enterobacteriales remained higher in children who did not respond to ferrous sulfate treatment compared to responders (p-value = 0.02) (Table 6.4, Figure 6.3). Higher relative abundance of Lactobacillales was negatively associated with recovering from anemia (p-value = 0.01) (Table 6.4, Figure 6.3). Bivariate testing between the relative abundance of Lactobacillales and covariates demonstrated an association between being female and having a higher relative abundance of Lactobacillales (p-value = 0.00) compared to males. While Clostridiales and Bifidobacteriales were positively associated with responding to iron supplementation, they did not become statistically significant (p-value = 0.91 and 0.52, respectively) in final models. T-tests demonstrated a relationship between Clostridiales and BMI Z-score (higher relative abundance in those classified as overweight, p-value = 0.03) and Bifidobacteriales and final CRP level (higher relative abundance in preschoolers classified as having low CRP, p-value = 0.05).

In bivariate models testing differences at the genus and family level, only one family-level taxa, Barnesiellaceae, was significantly higher in children who did not respond to treatment compared to responders in posttreatment samples (p-value = 0.00). In models controlling for pathogenic exposures, Barnesiellaceae remained significantly associated with response to iron supplementation (p-value = 0.02) (Table 6.5). T-tests show an association between higher relative abundance of Barnesiellaceae in children categorized as having low BMI z-score compared to those with a high BMI z-score (p-value = 0.01).

Additionally, regression models demonstrated a higher ratio of Bifidobacteriales to Enterobacteriales in children who responded to iron supplementation than non-responders (p-value = 0.06). However, this relationship did not remain statistically significant in models with pathogenic exposure co-variates (p-value = 0.28). Additional t-tests show a higher ratio of Bifidobacteriales to Enterobacteriales in preschoolers tested in the winter than in the summer (p-value = 0.03).

DISCUSSION

In this sample, only half of the anemic children had Hb levels above 11.0g/dL after one month of treatment with ferrous sulfate syrup despite high adherence to daily iron supplementation. Few children had indications of parasitic infection and responders were older and had higher BMI z-scores than non-responders. Seasonal differences were also observed, with more responders being interviewed during the summer months.

Like many of the aforementioned studies investigating the effects of iron supplementation on microbiota composition, we did not find differences in diversity between pre- and post-supplementation samples. Unlike previous studies, however, we found a higher level of Enterobacteriales at baseline compared to samples collected after one month of treatment (Paganini et al. 2016; Jaeggi et al. 2015; Zimmermann et al. 2010).

Additionally, we did not find significant differences in diversity between responders and non-responders within pre- and post-treatment samples. We did document some differences in the abundance of some microbial taxa at the phyla level between baseline and post-supplementation as well as between children who responded and did not respond to iron supplementation at both time points, but the majority of these findings were with taxa that had less than 1% relative abundance. In post-supplementation samples, relative abundance of Proteobacteria was greater than 1% and analyses demonstrated that responders had a lower number of Proteobacteria. This pattern remained when controlling for pathogenic exposures.

Studies exploring the association between an abundance of the phylum Proteobacteria, named after Greek prophetic sea-god Proteus, and intestinal microbiota dysbiosis have demonstrated the role of this micro-organism in establishing an unstable gut microbial community (Litvak et al. 2017) and is a potential diagnostic criterion for metabolic and nutritional diseases (Shin et al. 2015). In bivariate analysis, we documented an association between higher levels of Proteobacteria and geophagy. The consumption of dirt may increase a child’s exposure to pathogens (Geissler et al. 1998). Our findings support previous research documenting the association between blooms of Proteobacteria and poor health outcomes, such as Crohn's and inflammatory bowel disease (Morgan et al. 2012; Ogura et al. 2001, respectively).

At the order level, pre-supplementation samples had a higher relative abundance of Enterobacteriales compared to post-supplementation samples, but this finding did not remain statistically significant with the addition of pathogenic exposure variables. At baseline, there were no differences between responders and non-responders with a relative abundance greater than 1%. After one month of treatment, several order-level differences were observed between children who responded to treatment and those who did not. Two of these taxa remained significant in models controlling for variables representing exposure to pathogens. Children with a higher relative abundance of Enterobacteriales and Lactobacillales were less likely to respond to iron supplementation. A similar pattern was observed at the family-level within both pre- and post-supplementation samples with the taxa Barnesiellaceae.

Enterobacteriales is an order under the phyla Proteobacteria that requires iron for growth and virulence (Payne and Neilands 1988) and may play an important role in immune dysregulation (Bjorksten et al. 1999). While not all Enterobacteria are pathogenic, abundances of closely related species can predict the susceptibility to intestinal colonization by pathogenic bacteria (Zimmermann et al. 2010). Our finding that a higher abundance of Enterobacteriales is associated with a decreased likelihood of responding to treatment may be reflective of the immune response to inflammation produced by Enterobacteriales blooms in the intestine and/or reduced absorption of nutrients by the intestinal wall caused by gastrointestinal morbidity. This finding provides insight into the documented association between iron supplementation and the increased risk of diarrhea in children (Gera and Suchdev 2002).

Unlike Enterobacteria, Lactobacillales does not require iron and is seen as a beneficial barrier taxon within the gut due to its role in the prevention of colonization by enteric pathogens (Bezkorovainy et a. 1996) and its role in establishing intestinal mucosal layers that are crucial in maintaining gut barrier function (Qi et al. 2019). In our study we found that a higher abundance of Lactobacillales was associated with a lack of response to treatment in post-supplementation samples. This association may be driven by the increase of Enterobacteria, a higher number of enteric pathogens may lead to a greater number of Lactobacillales to maintain a healthy gut. A high abundance of Lactobacillales is an indicator of gut immaturity (Gritz and Bhandari 2015), which may reflect immaturity in other biological functions, like the immune system. A young immune system would not have developed specific immune defenses to pathogens and would reduce the body's ability to fight infection. Thus, a high abundance of Lactobacillales may represent immaturity in both the gut and immune development.

Studies investigating Barnesiellaceae are limited, McClorry et al. (2018) identified differences in Barnesiellaceae abundance between children who had iron deficiency anemia and reference participants. Another study demonstrated an association between reduced levels of this taxa and pediatric acute-onset neuropsychiatric syndrome (PANS), which includes conditions that impair brain neurologic function (Quagliariello et al. 2018). Future research investigating the relationship between anemia and cognitive function should consider incorporating theory and methods that address the gut-brain axis to further explore the association reported here.

Our results are limited by several important factors, the sample of anemic children who received iron supplementation is small (n=50). This small sample size may have limited power to find statistically significant differences between baseline and post-treatment samples as well as between responders and non-responders. Nevertheless, this study included the majority of pre-school-aged children in these San Juan de Lurigancho neighborhoods. Additionally, while Hb concentration is inexpensive and easy to measure with field-friendly testing, it lacks the specificity for establishing iron status (Balarajan et al. 2011).

Despite these weaknesses our study expands the geographic focus of iron supplementation studies in children and contributes an important case study to the literature on the health impacts of iron fortification. Through the use of an evolutionary medicine perspective as well as the inclusion of developmental microniche methods to create pathogenic exposure variables, this study provides evidence for the gut microbiome as a pathway that links iron supplementation and child recovery from anemia. Our study demonstrates an association between increased iron availability, intestinal dysbiosis, and a reduced likelihood of recovering from anemia, suggesting that increased iron intake with poor gut health may increase child morbidity. These findings lend support to evolutionary medicine theories on anemia and suggest that investigating pathogen exposure and microbial health is important to better understand the impact of iron fortification on child health and development. Future research is needed to untangle the relationship between exposure to pathogenic and nutritional exposures and microbiota composition. Nonetheless, our study highlights the importance of examining anemia and the microbiome across diverse contexts and the potential benefits of incorporating such work in the creation and implementation of anemia interventions.

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FIGURES

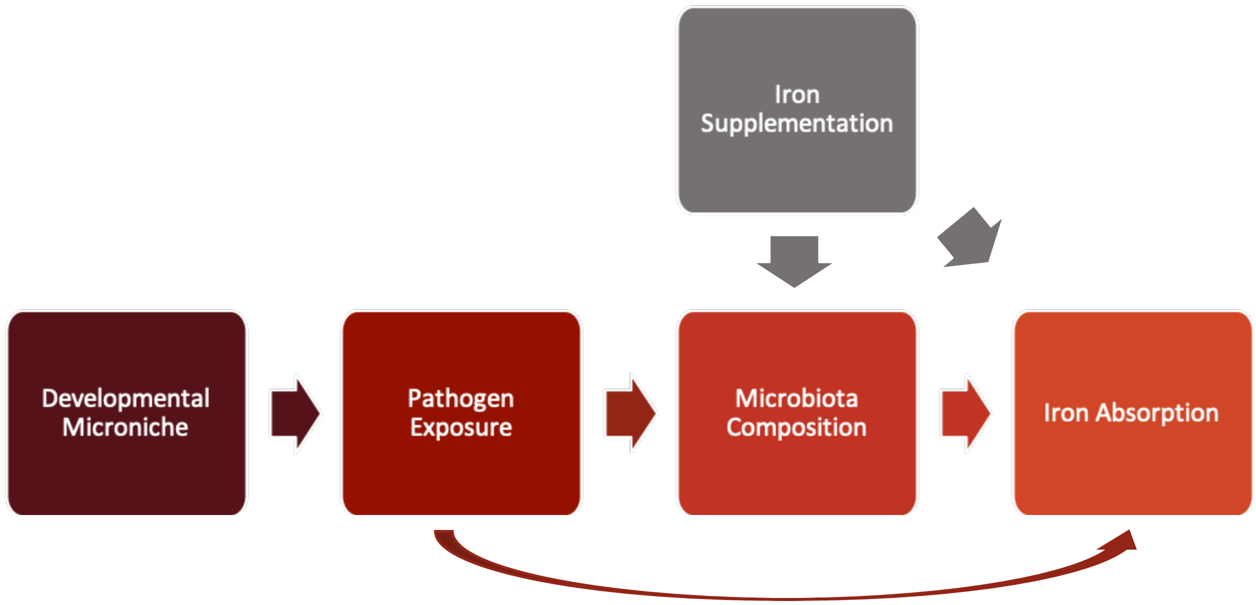


FIGURE 6.1: Conceptual framework for the associations between the developmental microniche and iron supplementation on intestinal microbiota composition and iron absorption

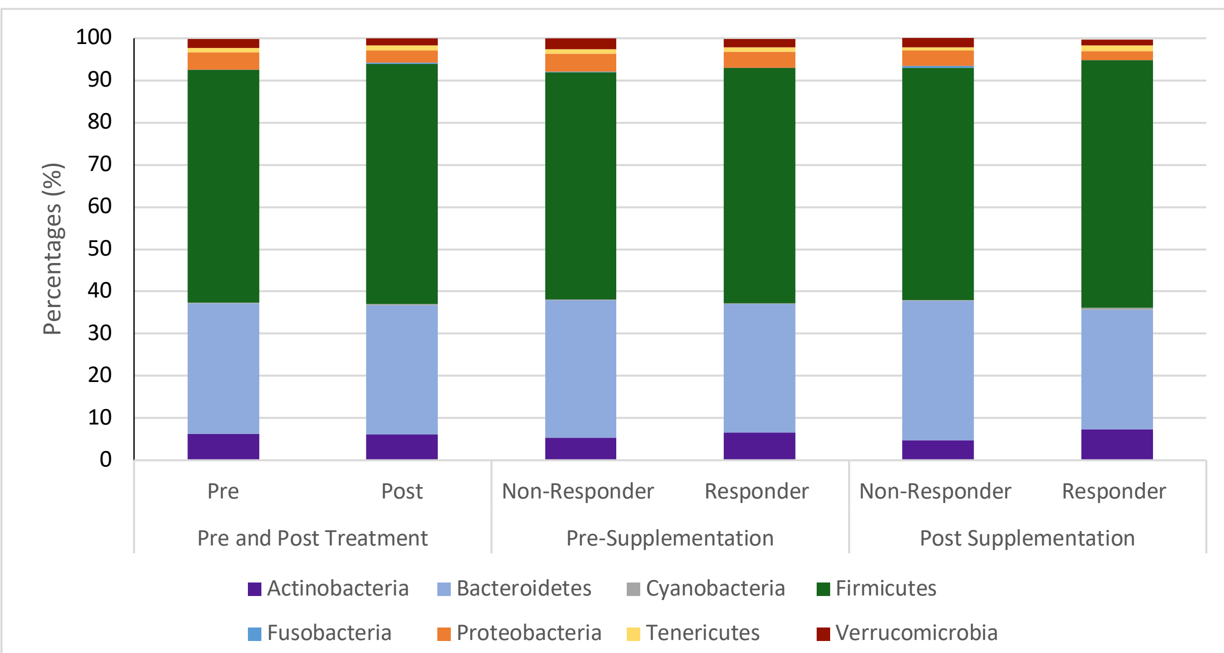
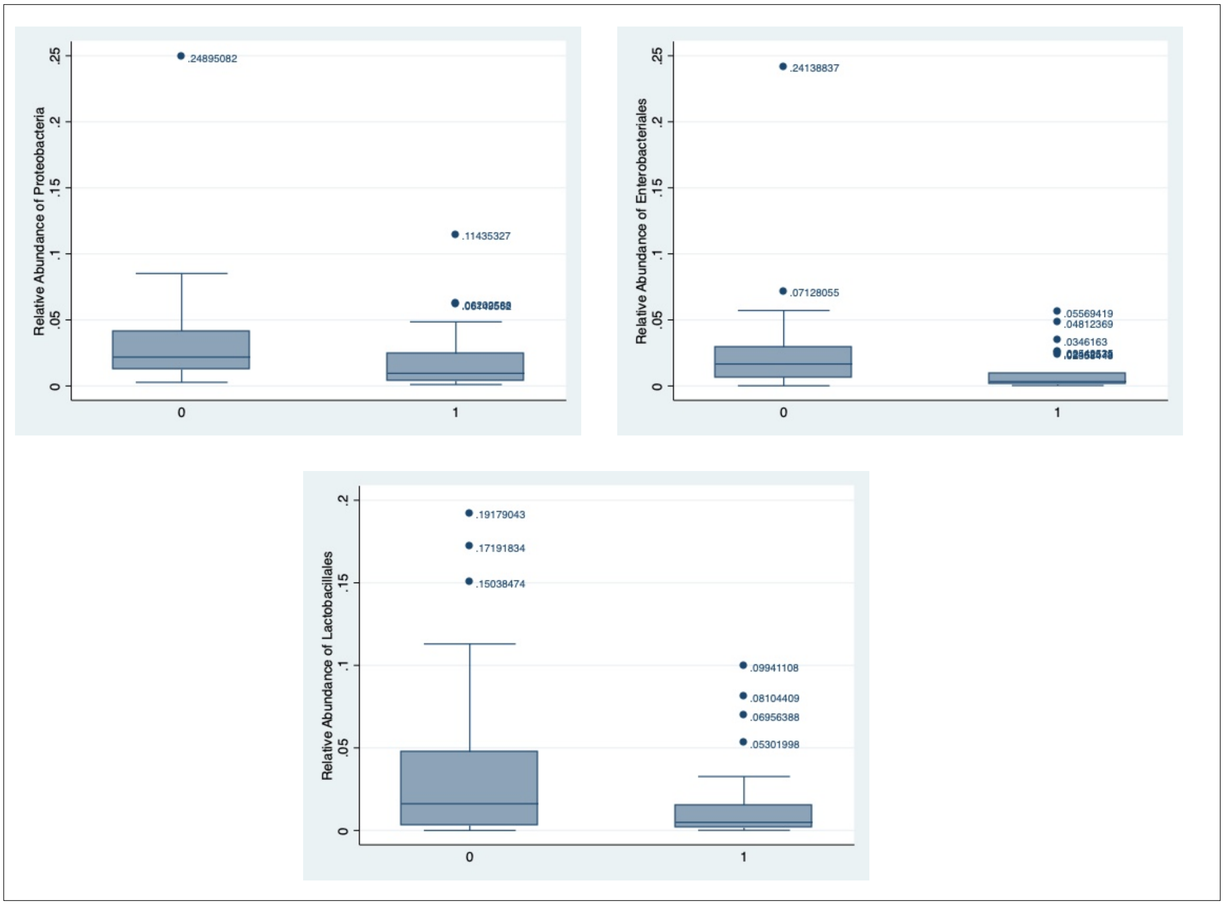


FIGURE 6.2: Relative abundance of phyla by timing of sample collection and response to iron supplementation



0 = Non-Responder, 1 = Responder

FIGURE 6.3: Differences in post-supplementation microbiota taxa by response and non-response to iron supplementation that remained significant in models with pathogen covariates

TABLES

TABLE 6.1: Descriptive characteristics for children who did and did not respond to treatment (mean and [SD] for continuous variables**)**

Variable	Responders	Non-Responders
n	25	25
Female	11 (44.0)	12 (48.0)
Age (years)	3.04 (1.17)	2.96 (1.02)
Mestizo	17 (68.0)	17 (68.0)
Adherence (>=22 days)	12 (48.0)	14 (56.0)
Parasite Presence	0 (0.00)	3 (12.0)
Season (summer)	13 (52.0)	3 (12.0)
CRP > 2.2 mg/L	5 (20.0)	10 (40.0)
Body Mass Index Z-Score >1.0	9 (36.0)	5 (20.0)
Consumption of Dirt	11(44.0)	5 (20.0)

TABLE 6.2: Phyla-level differences with <1% abundance

Between Pre and Post	Within Post	
> Post	*> in Responders*	*> in Non-Responders*
Spirochaetes	Spirochaetes	Fusobacteria
Fusobacteria	Lentisphaerae	
Cyanobacteria	Elusimicrobia	
	Cyanobacteria	

TABLE 6.3: Order-level differences with <1% abundance

Between Pre and Post		Within Pre	Within Post	
> in Pre	*> in Post*	*> in Responders*	*> in Responders*	*> in Non-Responders*
Anaeroplasmatales	Pseudomonadales	Brachyspirales	Brachyspirales	Neissariaes
Brachyspirales	Sphingomonadales	Aeromonodales	Campylobacterales	Rhizobiales
Campylobacterales	Caulobacterales	Campylobacterales	Victivallales	
Desulfovibrionales		Rhizobiales	Elusimicrobiales	
Fusobacteriales		Turicibacteriales		

TABLE 6.4: Multivariate regression models for response to iron supplementation by relative abundance of microbiota taxa in post-supplementation sample

Variables	Phyla (β) Proteobacteria	Order (β) Enterobacteriales	Clostridiales	Lactobacillales	Bifidobacteriales
Age (years)	0.07	0.06	0.08	0.08	0.08
Female	-0.06	-0.08	-0.06	0.05	-0.08
BMI Z-Score >1.0	*0.23	*0.22	*0.23	**0.25	*0.23
CRP > 2.2 mg/L	**-0.28	**-0.27	**-0.27	**-0.25	*-0.25
Season (Summer)	**-0.51	**-0.48	**-0.52	**-0.51	**-0.51
Consumption of Dirt (yes)	**0.37	**0.38	**0.31	**0.35	**0.31
Microbiota Taxa	*-2.71	**-3.62	0.04	**-3.19	0.90

Results for regression models for each taxon separately; all models account for clustering by maternal identification number
*p-value <0.10, **p-value <0.05

TABLE 6.5: Multivariate regression models for response to iron supplementation by Family: Barnesiellaceae in pre and post-supplementation samples

Variables	Pre-Supplementation (β)	Post-Supplementation (β)
Age (years)	0.08	0.09
Female	-0.27	-0.12
BMI z-score >1.0	0.21	0.13
CRP (> 2.2 mg/L)	**-0.32	-0.24
Season (summer)	***-0.50	***-0.50
Consumption of dirt (yes)	***0.33	**0.28
Barnesiellaceae	**-2.80	**-7.56

**p-value <0.05, *** p-value <0.01

CHAPTER 7. DISCUSSION

While anemia is a global health problem, effective strategies for anemia reduction require an understanding of context-specific causes and interventions that address predictors effectively. This dissertation aimed to contribute to our growing knowledge about anemia as well as iron supplementation among preschool aged children in a community within San Juan de Lurigancho.

KEY FINDINGS

Childhood Anemia

Half of all children in this sample were anemic, making the prevalence of anemia in this community higher than the national, Lima region, and Lima district rates (43.6%, 33.2%, and 26.4%, respectively) (ENDES 2017). The only area within Peru that reports an anemia prevalence higher than what is seen in this study is Puno, a city located near Lake Titicaca at an altitude of 3,830 meters (ENDES 2017).

The first goal in Chapter 4 was to explore the overall prevalence of anemia and to identify aspects of the developmental microniche that are associated with anemia status. While a number of variables were predictive of anemia status at the bivariate level, only three remained significant in the final model. I found that heavier participants, children living with their paternal grandparents, and those who enrolled in the study during the winter were less likely to be anemic than their counterparts.

Response to Iron Supplementation

In Chapter 4, I also considered how national level campaigns to reduce anemia are operate through socio-ecological factors at a more individual level by investigating the efficacy of iron supplementation within this particular context. Of the 50 children diagnosed with anemia at the beginning of our study, only 25 responded to iron supplementation treatment. This result is counter to the findings described in a review article that included a meta-analysis of 21 data sets from randomized-control-trials (RCTs) exploring iron supplementation in children 0 to 12 years-of-age (Ramakrishnan et al. 2004). Ramakrishnan et al. (2004) found a significant difference in the mean change in hemoglobin (Hb) concentrations between treatment and control groups (OR:1.49, CI:0.46-2.51, p-value<0.05). A more recent comprehensive review of the efficacy of iron supplementation concluded that the majority of RCTs investigating the effectiveness of iron treatment in children report significant increases in Hb concentration and other iron status indicators as well as reduced anemia prevalence (Iannotti et al. 2006). The differences between our current results and the findings presented in the aforementioned review articles may be due to variation in the developmental microniche of each child, specifically nutritional status and exposure to disease, difficulty in giving treatment due to child personality, and differences in the length of treatment.

Like anemia status, there were a number of socio-ecological factors that predicted response to iron supplementation at the bivariate level, but only three remained significant in the final model. A lower WAZ, an elevated CRP at the final interview, and being enrolled in the study during the summer season were associated with a reduced likelihood of a child responding to treatment.

When iron supplementation has not been effective in reducing anemia individually, experts suggest investigating poor compliance (Galloway and McGuire 1994) and malabsorption

(Lopez et al. 2016). Despite more than half of caregivers reporting adhering to the ferrous sulfate syrup regiment for 22 days or more, only half of the children enrolled in this study recovered from anemia at the end of one month of treatment, the standard clinical treatment protocol in Peru. This low rate of response to iron supplementation with high rates of adherence to treatment may demonstrate reduced iron absorption.

In Chapter 5, I explored potential pathways for reduced iron absorption, including immune activation and adiposity. Children who did not respond to treatment had higher rates of common cold symptoms and higher mean CRP. While the presence of cold symptoms was not associated with response to iron supplementation, high CRP reduced a child's odds of responding to iron supplementation.

Rates of overweight in the sample varied depending on adiposity measure, with the highest rates of overweight measured by WHtR and the lowest rates of overweight with BMI z-score. Higher WHtR and BMI z-score were associated with increased odds of responding to treatment, TSF z-score was not associated with response to iron supplementation in preliminary models and was excluded from models exploring my second hypothesis, that variation in body fat stores will moderate the association between immune function and response to treatment.

I found that both BMI z-score and WHtR moderate the interaction between CRP and response to iron supplementation, but the two adiposity measures had different probability patterns. Children with a high BMI z-score (in both high and low CRP groups) and those with low BMI z-score and low CRP all had greater than a 50% probability of responding to iron supplementation. Participants with low BMI z-score and high CRP were the least likely to respond to treatment.

While I expected a reduced probability of response to iron supplementation associated with high immune activation compared to low immune activation in children with low and high body fat, I observed a different pattern when exploring BMI z-score as a continuous variable. While children with low CRP were more likely to respond to treatment than participants with high CRP, lower BMI z-score was associated with decreased probability of responding to treatment. The odds of responding increased as BMI z-score approached zero in both CRP categories. The probability of responding continued to increase steadily for those with low CRP and dramatically for those with high CRP.

In this dissertation, I also investigated the role of the intestinal microbiota on response to iron supplementation and presented the results in Chapter 6. Like many of the studies investigating the effects of iron supplementation on microbiota composition, I did not find differences in diversity between pre- and post-supplementation samples. Unlike previous studies, however, I did find a higher level of Enterobacteriales at baseline compared to samples collected after one month of treatment (Paganini et al. 2016; Jaeggi et al. 2015; Zimmermann et al. 2010).

Additionally, I did not find significant differences in diversity between responders and non-responders within pre- and post-treatment samples. I documented some differences in the abundance of some microbial taxa at the phyla level between baseline and post-supplementation as well as between children who responded and did not respond to iron supplementation at both time points, but the majority of these findings were with taxa that had less than 1% relative abundance. In post-supplementation samples, relative abundance of Proteobacteria was greater than 1% and analyses demonstrated that responders had a lower number of Proteobacteria. In models with pathogenic exposure variables, this pattern remained.

At the order level, pre-supplementation samples had a higher relative abundance of Enterobacteriales compared to post-supplementation samples, but this finding did not remain statistically significant with the addition of pathogenic exposure variables. At baseline, there were no differences between responders and non-responders with a relative abundance greater than 1%. After one month of treatment, several order-level differences were observed between children who responded to treatment and those who did not. Two of these taxa remained significant in models controlling for variables representing exposure to pathogens. Children with a higher relative abundance of Enterobacteriales and Lactobacillales were less likely to respond to iron supplementation.

IMPLICATIONS

While Chapters 4, 5, and 6 investigate different aspects of childhood anemia and response to iron supplementation, similar patterns of predictors were observed across the studies for markers of inflammation, adiposity, and season. Two variables showed promise as predictors for child anemia and response to iron supplementation but were excluded due to the small sample size. Consuming heme sources of iron in the past six months is protective against anemia. This is due to the high bioavailability of iron in both red and organ meat and blood. The Peruvian diet is typically low in iron (Creed-Kanashiro et al. 2003). In this sample, caregivers discussed the lack of heme-source foods in their child's diet and often contributed the lack of these provisions to the child or their partner refusing to eat them and not liking the taste.

Another variable that was unable to be explored fully in this paper was caregiver reports on seeing their child eat dirt in the previous week. Eating dirt was significantly associated with child response to iron supplementation and in bivariate models geophagy was associated with a higher abundance of Proteobacteria and Enterobacteria. While consumption of dirt has been associated with iron status and anemia (Young 2011; Geissler et al. 1998), there is a lack of research on geophagy and iron supplementation response. Unfortunately, this study did not collect additional information about geophagy practices in children who were classified as not anemic and therefore, could not investigate the relationship between anemia status and eating dirt. Assessment of longitudinal data and the contents of the dirt in this area are needed to further explore the association between anemia status and geophagy.

C-Reactive Protein

In Chapter 4, while the initial level of CRP did not remain a significant predictor of response to iron supplementation, having an elevated CRP at the final visit remained significant significant with WAZ, maternal education, persons-per-bedroom ratio, and season covariates. Similarly, in Chapter 5, children who did not respond to treatment had higher rates of common cold symptoms and higher mean CRP. While the presence of cold symptoms was not associated with response to iron supplementation, high CRP reduced a child's odds of responding to iron supplementation. The different patterns observed between our subjective and objective measures of immune activation may be due to individual variation in reporting child morbidity symptoms or that CRP concentrations can be elevated despite a lack of observable symptoms (Panter-Brick et al. 2001; Rousham et al. 1998).

These results demonstrate an interesting relationship between levels of inflammation and anemia status. While earlier studies report opposing results on the association between morbidity and anemia (Wander et al. 2009; Hadley and DeCaro 2015), a more recent study investigating the association between infection and anemia status within the San Juan de Lurigancho community found that while morbidity was not predictive of anemia status over a six-month interval period, it was shown to be associated with current morbidity symptoms (Dorsey et al. 2018). These findings support investigating anemia status as an allostatic system that responds to infection adaptively, rather than expecting an optimal pre-infection value and demonstrates evolutionary medicine's potential to provide insight into patterns of recovery from anemia.

Adiposity

In both Chapter 4 and 5, heavier children were more likely to have a positive health outcome, they were less likely to be anemic and more likely to respond to iron supplementation despite using different measures of adiposity (WAZ, BMI z-score, WHtR). These statistical results were also reflected in the qualitative data, with mothers associating smaller body size with poor health and an increase in child appetite as a signal that they were recovering from anemia. This demonstrates that caregiver impressions of child body size play an important role in whether caregivers believed their child was healthy or not. These results are comparable to conclusions from previous research on infant feeding practices. A study conducted by Heinig et al. (2006) report ethnographic evidence from sixty-five Women, Infants and Children (WIC) eligible participants that demonstrate a positive association between higher infant weight and health in infants. Thompson and Bentley (2013) document that the cultural belief that "greedy" infants are healthier influence maternal feeding practices among first-time, low- income African-American mothers in central North Carolina participating in the Infant Care Study. The strong association between size and wellbeing in our sample may be due to cultural beliefs related to the aforementioned successful campaigns to reduce stunting in Peru. Only 19% of children in this sample were overweight, however, 49% of the sample were stunted. Stunting has been shown to be associated with increased risk of obesity in childhood (Wells et al. 2020; Popkin et al. 1996). Therefore, the positive association between larger body size and health may lead to difficulty in recognizing nutritional deficiencies as the child ages.

Results from previous research investigating the relationship between weight and anemia status are mixed. Some studies report that higher body mass index (BMI) results in an increased risk for iron deficiency and anemia among children and adolescents in both high income and transitioning settings (Aberli et al. 2011; Eftekhari et al. 2009; Zimmermann et al. 2008; Nead et al. 2004). While other studies have observed lower rates of anemia in women and children experiencing overnutrition (Kroker-Lobos et al. 2011; Eckhardt et al. 2008). Zimmerman et al. (2008) investigated the relationship between weight and anemia status as well as iron fortification. They report that increased adiposity in women and children results in lower anemia prevalence as well as a reduced response to iron fortification. In a study examining the efficacy of iron supplementation, Baumgartner et al. (2013) report that South African children with high BMI-for-age-z-scores have a greater risk for remaining iron-deficient after iron supplementation for 8.5 months when compared to children with low BMI-for-age-z-scores.

My results add evidence for the positive association between weight and anemia status but are contradictory to the findings on weight and iron supplementation presented by Zimmerman et al. (2008) and Baumgartner et al. (2013). The dissimilarity between our findings and the aforementioned studies may be due to differences in sample (e.g. age), study design, and diet. In Peru, where diet quality is likely to be poor (Creed-Kanashiro et al. 2003), the overweight children in this sample may have accrued enough iron or other minerals related to iron absorption (e.g. vitamin A and zinc) that lower their risk of anemia compared to non-overweight participants (Eckhardt et al. 2008). This contradiction may also be due to the complex set of environmental and individual variables that include differences in disease exposure and immune activation caused by specific economic and cultural contexts. More work is needed to understand the range of variation in inflammatory processes associated with central and peripheral adiposity.

Season

The summer season was associated with the more negative health outcomes, children enrolled in the study during the summer season were more likely to be anemic and less likely to respond to iron supplementation. Seasonal variation in anemia rates has been documented by several authors (Senn et al. 2010; Rogerson et al. 2000), however these studies link Hb fluctuations to increased rates of malaria during the rainy season in Papua New Guinea and Malawi, respectively. However, malarial parasites are not found in the urban areas of Lima and therefore not the cause of the seasonal patterns documented in this study. Seasonal distribution of rotavirus, a virus linked to severe gastroenteritis and a potential cause of anemia, has been documented in District of Independencia, but the prevalence of rotavirus peaked during the winter months in this peri-urban community within Lima and rates of this virus have decreased since the introduction of the rotavirus vaccine (Chang et al. 2015).

Fluctuations in diet may also contribute to the differences observed in anemia status and response to iron supplementation between the winter and summer months. While seasonal variation in diet has been documented in highland Peru, children experienced little seasonal change in in energy in-take (Leonard and Thomas 1989). However, a study investigating child (0-35 months) nutritional status in Pampas de San Juan de Miraflores from 1987-1993, report seasonal variation, the mean weight-for-height was an estimated 0.38 z-score higher in the winter than in the summer (Marin et al 1996). The negative outcomes associated with the summer season (anemia and non-response to supplementation) may be linked to significant variation in nutritional status.

While markets provided a wide-range of foods all year round, children were enrolled in school during the winter months. Based on the preliminary investigation of 24-hour dietary recalls, diet patterns in the summer months were un-structured compared to the winter season.

Meal times varied day to day and snacks were more frequent. In the winter, all children in the sample were enrolled in pre-school, and the days became more structured. Breakfast and lunch were dictated by the start and end of the school day. Seasonal variation in anemia and response to treatment in our sample may be due to differences in the composition and structure of child diet related to enrollment in school. Eating sporadically and less frequently may cause depletion of vital nutrients and minerals, resulting in higher rates of anemia during the summer.

Additionally, alterations in intestinal microbiota caused by fluctuations in pathogenic exposure and diet may also serve as a pathway linking season and negative health outcomes. The finding that a higher abundance of Enterobacteriales is associated with a decreased likelihood of responding to treatment may be reflective of the immune response to inflammation produced by Enterobacteriales blooms in the intestine and/or reduced absorption of nutrients by the intestinal wall caused by gastrointestinal morbidity. Furthermore, I also documented an association between higher levels of Proteobacteria and Enterobacteria in the stool samples collected during the summer season than in those collected during the winter months in bivariate analysis. Additional investigation is needed to determine if the driving force of the seasonal variation observed in this dissertation is due to disease ecology.

LIMITATIONS

This study investigates a large range of child, maternal, household, and environmental factors that that allow for an in-depth investigation of predictors for anemia and response to iron supplementation and test more directly the association between children's biology and socio-ecological context. However, the results are limited by several important factors and should be considered preliminary.

The results are limited by several important factors, the sample of anemic children who received iron supplementation is small (n=50). This small sample size resulted in large confidence intervals in logistic regression models and may have limited power to find statistically significant differences between responders and non-responders.

Despite Hb being an inexpensive and easy to measure with field-friendly testing, it lacks of specificity for establishing nutritional anemias, such as iron status. Additionally, while some studies suggest that Hb may not be affected by iron supplementation and recommend including measures of iron status (Stolzfus et al. 2004), others report increases in Hb but not in markers of iron status (serum ferritin and free erythrocyte protoporphyrin) in participants receiving daily iron supplementation (Zavaleta et al. 2000). Due to the conflicting reports on iron supplementation's impact on Hb status, future research should explore the effects of iron supplementation on additional biomarkers associated with anemia and iron level, such as serum transferrin receptor (sTfR).

Another limitation of our study is the use of a single inflammation measure, CRP. To further explore the association between adiposity, immune activation, and response to iron supplementation a variety of pro-inflammatory cytokine biomarkers, such as IL-6 and TNF-α, should be used in future research.

RECOMMENDATIONS

Despite these limitations, these findings highlight the importance of incorporating a developmental microniche perspective and methodology in research investigating childhood anemia and response to iron supplementation. Factors that affect the prevalence and distribution of anemia in a population involve the complex interplay of political, ecological, social, and biological factors. Given the persistent nature of childhood anemia in Peru and the failure of interventions focusing on the iron supplementation and fortification strategies, understanding the roles of the developmental microniche and factors that can be modified to improve nutritional status and disease exposure is a critical step in reducing childhood anemia prevalence.

These results also further our understanding of the relationship between immune activation and anemia status within a dual burden context. This study demonstrates Life History Theory's potential to provide insight into patterns of disease and highlights the need for further investigation of child inflammatory profiles within a dual burden context. The human immune system is characterized by substantial developmental plasticity. Longitudinal research on immune function demonstrates nutritional and microbial exposures in early childhood are important determinants of inflammation in adulthood (McDade 2012). The inclusion of central and peripheral adiposity measures in this study expands our knowledge on the influence of visceral adipose tissue on the relationship between immune function and anemia in children. Further research on the variation of inflammatory processes associated with visceral adiposity in childhood is needed to investigate pathways to health and disease later in life.

This study also expands the geographic focus of iron supplementation studies in children and contributes an important case study to the literature on the health impacts of iron fortification. Through the use of an evolutionary medicine perspective as well as the inclusion of developmental microniche methods to create pathogenic exposure variables, this study provides evidence for the gut microbiome as a pathway that links iron supplementation and child recovery from anemia. These findings suggest that investigating pathogen exposure and microbial health is important to better understand the impact of iron fortification on child health and development. Future research should investigate the relationship between pathogenic and nutritional exposures and microbiota composition.

This research has important public health implications. The associations between child growth patterns coupled with maternal perceptions of child body size, household composition, and seasonal variation with anemia status and response to iron supplementation presented in this paper, indicate the importance of including analysis of caregivers, household, and environmental-level variables in addition to individual-level characteristics in studies of childhood anemia. While the probability of anemia and overweight co-occurring may be low, both of these conditions are caused by malnutrition and have links to chronic disease and negative developmental effects (Stoltzfus et al. 2004; Popkin et al. 2006). The high prevalence of anemia and the rising rates of overweight and obesity in Peru warrant prevention and education efforts as well as further investigation into the dual burden of disease. This study also highlights the importance of examining anemia and the microbiome across diverse contexts and the potential benefits of incorporating such work in the creation and implementation of anemia interventions.

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APPENDIX A: SERUM TRANSFERRIN RECEPTOR

SAMPLE COLLECTION

I recruited nine additional participants to donate their blood and time to my dissertation research at the Human Biology Laboratory at the University of North Carolina – Chapel Hill (UNC). The six men and three women were all UNC affiliates and 89% were graduate students. Three samples were collected from each participant; fingerstick dried blood spots, plasma, and venipuncture dried blood spots.

Fingerstick Dried Blood Spots

The participants' finger was cleaned with alcohol, and a sterile disposable microlancet was used to deliver a controlled puncture. At least one drop of blood was collected from participants after the finger prick on standardized filter paper (Whatman #903. Middlesex, UK). Once the blood spots were dry, I placed them in the -20°C freezer until I began laboratory analysis.

Venipuncture Dried Blood Spots

Isaura Godinez conducted all phlebotomy procedures using the established and proper protocol. After collecting the vial of whole blood, I extracted 50 uL of blood using a pipette and deposited the collected liquid onto standardized filter paper (Whatman #903. Middlesex, UK). I performed this step four additional times to create a total of five blood spots. Once the blood spots were dry, I placed them in the -20°C freezer until I began laboratory analysis.

Plasma

To create plasma samples, I extracted roughly 25 mL of whole blood from the phlebotomy vials using a pipette and deposited the liquid into a properly labeled and clean plastic screw-cap vial. I placed the vials into a centrifuge for 15 minutes at 2500 RPM. I then pipetted the plasma into a second clean plastic screw-cap vial and labeled the vial. I stored plasma samples in the -20°C freezer until I began laboratory analysis.

SPIKE AND RECOVERY TEST

Sample and Spike Preparation

Elution Buffer: A glass container was rinsed with de-ionized water and let dry. 100 mL of PBS and 50 uL of Tween-20 were added to the glass container (McDade and Shell-Duncan 2002; Cook et al.1998). The solution was mixed thoroughly by pipetting beneath surface multiple times and then placed and secured on a plate rotator for 1 hour. The elution buffer can be stored in a 4°C refrigerator up to 30 days.

Spike and Recovery Test Samples: Three disposable tubes were labeled: un-spiked, spiked, and control. Mixed elution buffer was pipetted into the tubes as follows:

- Un-spiked: 100 uL
- Spiked: 98 uL
- Control: 98 uL.

To "spike" the elution buffer, 2 uL of the highest control (included in kit) was added to the spiked and control tubes. All three tubes were covered and placed on the plate rotator for 1 hour. They were then placed in a 4°C refrigerator overnight.

Protocol for Spike Test

Preparation: The day of the assay, the un-spiked, spiked, and control samples were removed from refrigeration and were placed on the plate rotator for 1 hour.

Spike and Recovery Dilutions: Three disposable tubes were labeled 1:2, 1:4, 1:8 for un-spiked dilutions. The dilutions were created for the un-spiked solution by adding 50 uL of assay diluent to each un-spiked dilution tube (1:2, 1:4, 1:8) and:

- 1:2 – adding 50 uL of unspiked solution and vortexing for 30 seconds
- 1:4 – adding 50 uL of 1:2 solution and vortexing for 30 seconds
- 1:8 – adding 50 uL of 1:4 solution and vortexing for 30 seconds

This protocol was repeated for both the spiked and control solutions.
After creating each dilution series, the sTfR kit was performed by following the Quantikine IVD ELISA Human sTfR (R&D Systems) instructions.

TABLE A.1: Plate lay-out for spike and recovery test.

	1	2	3	4	5	6
A	Standard		High Control		Un-spiked Sample 1:8	
B	Standard		Spiked Sample		Control Spike	
C	Standard		Spiked Sample 1:2		Control Spike 1:2	
D	Standard		Spiked Sample 1:4		Control Spike 1:4	
E	Standard		Spiked Sample 1:8		Control Spike 1:8	
F	Standard		Un-spiked Sample			
G	Low Control		Un-spiked Sample 1:2			
H	Mid Control		Un-spiked Sample 1:4			

Note: I did not have enough sample in the un-spiked, spiked, or control tubes to do replicates.

VALIDATION PROCEDURE

Elution Protocol

Sample: The day before an assay was performed blood spot samples from finger-stick and venipuncture were removed from the freezer and two discs of each were punched out using a standard 3.2mm (1/8 inch) hole punch and placed in disposable tubes. Each disc contains 1.15 uL of serum, this corresponds to a dilution factor of 1:33.3. Elution buffer (100 uL) was added and the tubes were covered and vortexed for 30 seconds.

Protocol for Immunoassay

Sample Preparation: The day of the assay, the samples and plasma were removed from refrigeration and were placed on the plate rotator for 1 hour.

Immunoassay: The sTfR kit was performed by following the Quantikine IVD ELISA Human sTfR (R&D Systems) instructions.

TABLE A.2: Plate lay-out for serum transferrin receptor validation procedure.

	1	2	3	4	5	6	7	8	9	10
A	Standard		High Control		Grad 08 - Plasma		Grad 07 - V DBS		Grad 06 - FS DBS	
B	Standard		Grad 01 - Plasma		Grad 09 - Plasma		Grad 08 - V DBS		Grad 07 - FSDBS	
C	Standard		Grad 02 - Plasma		Grad 01 -V DBS		Grad 09 - V DBS		Grad 08 - FS DBS	
D	Standard		Grad 03 - Plasma		Grad 02 - V DBS		Grad 01 - FS DBS		Grad 09 - FS DBS	
E	Standard		Grad 04 - Plasma		Grad 03 - V DBS		Grad 02 - FS DBS			
F	Standard		Grad 05 - Plasma		Grad 04 - V DBS		Grad 03 - FS DBS			
G	Low Control		Grad 06 - Plasma		Grad 05 - V DBS		Grad 04 - FS DBS			
H	Mid Control		Grad 07 - Plasma		Grad 06 - V DBS		Grad 05 - FS DBS			

*V DBS = Venipuncture dried blood spot sample
*FS DBS = Fingerstick dried blood spot sample

RESULTS

Recovery Calculation

The percent of recovery was calculated by:

- Percent Recovery = ((observed-un-spiked) / expected) x 100

Observed: Spiked sample
Un-Spiked: Un-spiked sample
Expected: Ratio of spike to diluent multiplied by control amount

The acceptable range of recovery is 80-120%. If the control spike is not within 80-120% range then there is an issue with the elution buffer.

Note: For sTfR assay the expected ratio is 2/98 x 80 = 1.633
My control spike was 0.00 because 1.633 is less than 3.0, the detection range of sTfR for the kit.

sTfR Recovery Calculation: ((13.266 – 11.435)/1.633) x 100 = 112%

Analysis of Assay Performance

Correlation: I performed a Pearson correlation test to see if the for the following combinations were correlated.

- Plasma and Venipuncture DBS
- Plasma and Fingerstick DBS
- Venipuncture DBS and Fingerstick DBS

TABLE A.3: Pearson correlation results for analysis of serum transferrin receptor validation procedure

	r (p-value)	Notes
Plasma and V DBS	0.64 (0.06)	Strong correlation, not significant
Plasma and FS DBS	0.41 (0.27)	Moderate correlation, not significant
V DBS and FS DBS	0.74 (0.02)	Strong correlation, significant

Mean Differences: I performed t-tests to explore whether or not the mean differences for the following combinations were significantly different.

- Plasma and Venipuncture DBS
- Plasma and Fingerstick DBS

*All paired differences should be within a 2-standard deviation range around the mean, this indicates an acceptable level of agreement.

TABLE A.4: Mean differences between serum transferrin receptor levels in plasma and two types of dried blood spots.

	Mean (SD)	t (p-value)
Plasma	1.08 (0.33)	-------------
V DBS	0.23 (0.11)	9.41 (0.00)
FS DBS	2.56 (0.92)	-5.29 (0.00)

NOTE: Grad 01 is outside of the 2 standard deviation range for comparisons between plasma and both dried blood spot samples.

LIMITATIONS

The small sample size may have limited power to find statistically significant differences between types of samples.

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